CENTRE OF EXPERTISE FOR WATERS

Review of monitoring techniques and sampling strategies to identify the most significant sources of faecal indicator organisms (FIO) within a catchment

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Executive Summary

Questions

1. Which current monitoring technology has been used successfully as part of field investigations to identify major sources of Faecal Indicator Organisms (FIO) within catchments, i.e. FIO hotspots?
2. Which emerging technologies are likely to be applicable and practical for identifying FIO hotspots?
3. How often would Scottish Environment Protection Agency (SEPA) need to sample to identify FIO hotspots and verify FIO modelled exports given storage and monitoring resource limitations in remote areas and complex river networks[1]?
4. What monitoring strategy would SEPA need to apply to address the pressure profile within the area of influence (aka zone of influence)?[2]
5. What is the timing of FIO discharges expected from each FIO source type, e.g. septic tank systems (STS), combined sewage overflows (CSO), storm-tank overflows (STO), wastewater treatment works (WwTW), wildlife and farmland?

Background

SEPA plan to use "blitz" monitoring to get a picture of water quality across catchments where there are multiple sources of faecal pollution to Bathing Water Protected Areas (BWPA) and Shellfish Water Protected Areas (SWPA). This is envisaged to involve FIO sampling across the river network to identify the area of influence, and trace FIO hotspots and types of sources within the area of influence. However, blitz monitoring is faced with a wide range of challenges, such as monitoring resource limitations, regulatory requirements for storage time and analytical procedures, and limited understanding where the area of influence and FIO hotspots are located. Addressing these challenges is essential for addressing the impacts of catchment-based faecal pollution to BWPA and SWPA.

Research undertaken

We undertook a literature review summarising best available evidence on the timing of FIO discharges, in-stream FIO variability, FIO pollution risk, FIO monitoring and detection technologies. We developed a desktop approach to identifying potential FIO hotspots. We also developed recommendations for a practical monitoring strategy to identify the area of influence to BWPA and SWPA, and to track FIO from different FIO hotspots and types of sources within it.

Key findings

- There is sufficient understanding of the broad factors determining timing of FIO discharges from different types of sources, in-stream FIO variation and FIO pollution risk across a river catchment.
- There is consensus among experts on the monitoring strategy needed to identify the area of influence and FIO hotspots therein as well as to differentiate between types of sources (i.e. human vs animal) within the area of influence.
- Current FIO technologies successfully used for FIO catchment investigations in the lab include cultivation-based methods (e.g. membrane filtration, Coliscan Easy gel system and Colilert); DNA-based methods (e.g. qPCR); biomarkers (e.g. sterols); or chemical tracers (e.g. caffeine, saccharin).
- Current FIO technologies successfully used for FIO catchment investigations in the field include using mobile labs after sample collection (without storage time) (e.g. Aquaflex and Colitag) and probes for proxy measurements (e.g. turbidity, conductivity, ammonia and temperature), or using *in situ* devices for continuous measurements (autosamplers).
- There is limited published information for the use of emerging FIO technologies in FIO catchment investigations. Technologies that could possibly be applied include:
    - o In the lab after field sample collection (e.g. RNA biosensors, Flow cytometry and Fluorescent Activated Cell Sorting, Paper-Origami DNA microfluidics and DNA-based methods for microbial source tracking (MST) such as microarray).
    - o In the field, probes (e.g. Bacti-Wader, aquaCHECK365, Bactiquant Water, Microbial Bioanalyser), or continuous monitoring technologies based on the detection of enzymatic activities (e.g. BACTcontrol).
    - o Emerging technologies are most powerful when used in combination with current technologies (e.g. aquaCHECK365 applied in combination with Colitag or turbidity sampling).
- Frequency of sampling for a given current or emerging FIO technology depends on the purpose of

1 No source-apportionment needed.

2 Here, this refers to the part of the river catchment in which diffuse and point FIO pollution sources can influence water quality in bathing water protected areas (BWPA) and shellfish water protected areas (SWPA).

sampling and knowledge of in-stream FIO variability at different scales at a given site and time. However, sampling frequency per FIO technology remains briefly addressed in the literature.

- The monitoring strategy to detecting catchment-based FIO sources involves three phases:
    - o Phase 1 identifies the area of influence and FIO hotspots therein through field surveys and monitoring with a desk-based initial screening component in data-rich catchments.
    - o Phase 2 studies in-stream FIO variability in relation to rainfall-dependent/-independent discharges from FIO hotspots in the area of influence through monitoring and modelling.
    - o Phase 3 involves monitoring in the area of influence to elucidate predominant types (i.e. human vs animals) of diffuse FIO sources using microbial and chemical source tracking tools.
- FIO discharges may be rainfall-dependent (e.g. CSO, STO and farmland runoff) or rainfall-independent (e.g. WwTW and STS effluent, artificial drains, livestock, wildlife, and leaching from STS soakaways).
- Temporal variability of in-stream FIO concentrations may be diurnal, storm event-scale, seasonal and interannual.

Recommendations[3]

Phase 1: Apply a toolbox approach integrating desktop studies, field monitoring and modelling:

1. Use the desktop screening approach developed here to identify potential FIO hotspots, e.g.:

- Point sources such as CSO, STO, WwTW serving more than 5000 people or tourist resorts; high-density STS clusters (>20 STS/km^2) and STS within 10 to 50m from watercourses located on soils at high runoff risk/ leaching potential.
- Diffuse sources including modelled areas of high in-stream FIO risk from livestock

2. Apply mobile lab technologies such as Colitag and aquaCHECK365 in combination with turbidity[4], temperature and flow to verify locations and FIO pollution from each potential FIO hotspot identified in the desktop study, as follows:

- Start from the waterbody catchments adjacent to BWPA or SWPA (i.e. coastal catchments).
- Prioritise human FIO hotspots (i.e. CSO, WwTW, STO, STS) or stream-river confluence sites draining areas influenced by human FIO sources in the coastal waterbody catchments.
- Inspect area for FIO risk from unmapped STS, wildlife, pets and other diffuse sources (e.g. streambed and streambanks) and verify their inputs.
- Select sampling sites that clearly link to known FIO sources, are wildlife-free when sampling, and display small variability during baseflow.
- Collect samples in short periods of time during wet and dry conditions (hybrid monitoring design) to address variability from rainfall-dependent and rainfall-independent discharges.

3. Identify the upstream limit of FIO pollution through monitoring upstream and downstream ("bracketing") potential FIO hotspots until a "clean" sample indicates no FIO impact from upstream. The area of influence may be sought upstream from the coastal waterbody catchments.

Phase 2: Apply membrane filtration techniques and flow cytometry in the lab or use mobile labs (e.g. Colitag) or continuous monitoring devices (e.g. ALERT – *E. coli* Analyser) to assess temporal variability of in-stream FIO (area of influence) concurrently with turbidity, temperature and flow.

Monitoring can be:

- Hourly for a day or two upstream and downstream of continuous human (e.g. WwTW and STS clusters) and/or animal (e.g. livestock farmland) FIO discharges during wet and dry days.
- Weekly or twice weekly (bi-weekly) for as long as necessary to understand discharges from CSO, STS clusters, and stream-river confluence sites.
- Event-scale to study the effects of rainfall-dependent FIO discharges such as CSO, STO and farmland runoff. Event-scale data can be redrawn from weekly time series.

Phase 3: Apply microarray, qPCR of genetic markers or flow cytometry for MST to track predominant FIO sources at sites influenced by diffuse FIO sources or mixed land use. This sampling is confirmatory or hypothesis-driven based on the evidence from Phase 1 and 2 on in-stream FIO variability downstream of CSO, STS clusters, confluence sites and at BWPA/SWPA. Sampling for MST can target wet and dry conditions or be one-off.

3 Our recommendations regarding the choice of monitoring technologies are based on the requirements from SEPA and do not preclude the use of the technologies that were not recommended for other purposes.

4 For detecting wastewater downstream of point sources and not as a surrogate for FIO, unless preliminary data suggest that turbidity correlates significantly with levels of FIO in the study area.

1.0 Introduction

The Scottish Environment Protection Agency (SEPA) seek to develop a practical in-stream microbiological monitoring programme to investigate catchment-based sources of faecal pollution to bathing water protected areas (BWPA) and to shellfish water protected areas (SWPA). Developing meaningful monitoring programs for managing microbiological water quality in BWPA and SWPA requires understanding of the factors influencing transport of faecal microorganisms from sources to BWPA and SWPA. Here, we review available evidence on the factors determining faecal microbiological pollution across a river catchment. We also outline strategies about how, how often and where to collect microbiological samples in-stream to identify sources of faecal pollution to BWPA and SWPA and to assess contribution from different types of faecal sources (i.e. animal versus human).

1.2 The problem

The regulatory framework controlling catchment-based sources of faecal pollution to BWPA and SWPA arises from The Water Environment and Water Services (Scotland) Act 2003 (as amended), aka WEWS Act, which transposes the Water Framework Directive (WFD) (2000/60/EC) to national law and is detailed in Appendix I.1. In brief, under the WEWs Act SEPA must assess and address the pressures impacting the water quality in BWPA and SWPA through the development of six-yearly River Basin Management Plans (RBMP) with the aim to achieve good classification status (or, if this is not possible, to reduce pressures) by 2027. In the context of the WEWS Act, SEPA also controls rural diffuse pollution and direct (point-source) effluent discharges to the water environment under the Water Environment (Controlled Activities) (Scotland) Regulations 2011 (as amended), also known as CAR. Further, the priority catchment approach, which was launched in 2011, helps SEPA to prioritise action in delivering the objectives set under the RBMP process (DPMAG-SEPA 2017). Regulations for water quality monitoring to inform status classification and the RBMP process are also in place. Classification is based on monitoring of faecal indicator organisms (FIO): BWPA classification is based on the presence of *Escherichia coli* (*E. coli*) and intestinal enterococci (IE) in water samples collected in bathing waters; SWPA classification is based on shellfish *E. coli* samples (Table 1).

The regulatory framework also includes provisions for catchment surveys to assess faecal pollution pressures in the catchments draining to BWPA (BW catchments) and to SWPA (SW catchments) (see Appendix I.2). Bathing water profiles are produced to provide evidence on types of faecal pollution pressures in the catchments draining to BWPA (BW catchments). In parallel, sanitary surveys in SWPA are undertaken to detect faecal pollution sources in coastal catchments draining to SWPA (SW catchments). In addition, experts from across the European Union (EU) Member States have provided guidance on how to produce bathing water profiles (Appendix I.3) and how to undertake sanitary surveys in SWPA (Appendix I.4).

The majority of BWPA in Scotland are at good or excellent status. However, there are still failing BWPA in both river basin districts of Scotland (SEPA 2015a; b). As of 2018/2019 bathing season (based on four years of monitoring data from 1 June to 15 September), 24 out of 86 BWPA fail the standards for good status (SEPA 2018). As reported in the bathing water profiles published on SEPA's web site (SEPA n.d.a), BWPA failing good status are subject to short-term pollution when heavy rainfall washes bacteria into the sea from the following major types of sources:

- Diffuse pollution sources: e.g. agricultural and urban run-off, and septic tank systems (STS).
- Point sources, e.g. treated effluent, combined sewer overflows (CSO), emergency overflows (EO).
- Overflowing public and private waste-water treatment works (WwTW).

Table 1. FIO standards for the classification of BWPA and SWPA to assess compliance with the objective of good status. Key: cfu: Colony Forming Units; MPN: Most Probable Numbers; FIL: Flesh and Intervalvular Liquid. Source: BW Regulations 2008; SG Directions 2015; 2016.

Classification	Thresholds *E. coli*, IE (cfu/100ml)		Confidence level
	Coastal Bathing Waters	Inland Bathing Waters	
Excellent	*E. coli:* ≤250; IE: ≤100	*E. coli*: ≤500; IE: ≤200	95th percentile
Good	*E. coli:* ≤500; IE: ≤200	*E. coli*: ≤1000; IE: ≤400	95th percentile
Sufficient	*E. coli:* ≤500; IE: ≤185	*E. coli*: ≤900; IE: ≤330	90th percentile
Poor	means that the values are worse than for Sufficient status		
	Aquaculture Shellfish Waters		Confidence level
Good	*E. coli:* ≤230 MPN /100g of FIL		90th percentile
Fair	*E. coli:* ≤4,600 MPN /100g of FIL		90th percentile
Insufficient	*E. coli:* >4,600 MPN /100g of FIL		90th percentile

- STS directly discharging into the river network or to soakaways near watercourses.
- Wildlife and other animal sources, e.g. pets.

On the other hand, faecal pollution pressures in SW catchments are less understood by SEPA than pressures in BW catchments, partly because the regulatory frameworks for undertaking sanitary surveys and supporting the RBMP process are not aligned under the WFD (Akoumianaki *et al*., 2018). As of 2014, only 28 out of 84 SWPA complied with the standards for good status. It must also be noted that water quality classification in SWPA is based on three-years' worth of monthly shellfish *E. coli* data collected by FSS in Shellfish Protected Areas (SPA) sitting within SWPA.

This robust regulatory framework underpinned by the WFD has a key limitation. Whilst regulatory FIO monitoring for classification at BWPA and SWPA alone can tell whether there is a faecal pollution or not, it is not enough to inform the RBMP process within BW and SW catchments (see Box 1). In addition, stormflow can export up to 98% of the annual *E. coli* load from agricultural catchments but represent only 6–30% of regulatory FIO monitoring samples (Muirhead, 2015; McKergow and Davies-Colley, 2010). Stormflow can also reduce in-stream FIO through dilution (e.g. Kay *et al*., 2008a), further perplexing understanding of the timing of FIO discharges to BWPA and SWPA.

Box 1. What FIO regulatory monitoring cannot do in the context of the RBMP process.

- Identify the locations of FIO sources (Domingo et al., 2007).
- Identify FIO sources, i.e. catchment-based versus marine sources (Domingo et al., 2007; Kinzelman and Ahmed 2016); or human versus animal sources (e.g. Nnane et al., 2015; Reisher et al., 2010).
- Address the timing of catchment-based FIO discharges, such as: rare EO discharges; rainfall-dependent CSO discharges; FIO from streambed sediments during baseflow; wildlife FIO; continuous discharges from STS and farmland (Byappanahalli et al., 2003; Ashbolt et al., 2010).

SEPA plan to use blitz monitoring to get a picture of water quality across a catchment where there are multiple diffuse pollution sources (B. McCreadie-SEPA, pers. com., May 2019). SEPA have envisaged blitz monitoring as follows:

"Blitz involves sampling at lots of points along a waterbody to trace where pollution is coming from. The sampling is carried out over the shortest time span and ideally on consecutive days. Microbiological surveys are usually carried out during wet weather and may be followed up by more detailed sampling in areas which require further investigation. This can help point to sources of diffuse pollution from livestock, septic tanks or sewer networks and help SEPA to target actions."

However, blitz monitoring is faced with a wide range of challenges:

- The part of the river catchment in which diffuse and point FIO pollution sources can influence water quality in bathing water protected areas (BWPA) and shellfish water protected areas (SWPA) is known as the area of influence or zone of influence. The upper limit of the area of influence is understood to be at the head-of-tide region (i.e. farthest point upstream where a river is affected by tidal fluctuations). However, in catchments with extensive sources of faecal pollution the area of influence may extend further upstream. **What monitoring strategy is fit for delineating the upper limit of the area of influence?**
- Microbiological field surveys are resource demanding, especially in remote areas (e.g. upland areas, and W. coast of Scotland) and across a complex waterbody network with several types of temporally variable point and diffuse FIO pollution sources. ***When, where and how often is in-stream FIO monitoring required?***
- Collecting multiple FIO samples in-stream to capture variability in FIO transport pathways and to track the origin of FIO in remote areas requires practical alternatives to laboratory-based analytical methods and, for laboratory analyses, to the storage time specifications. ***Are alternative monitoring techniques and technologies robust and feasible in the Scottish context?***
- Monitoring the impacts from septic tank systems (STS) is complicated because of uncertainties regarding the locations and characteristics of domestic STS (e.g. ground conditions, type of soakaway, age, emptying frequency and fate of sludge, and treatment and fate of effluent) remain poorly reported in Scotland (Brian McCreadie, SEPA, pers. com, December 2018). ***How can FIO monitoring account for STS discharges?***
- Identifying the area or zone of influence in BW and SW catchments (Appendix I.5; see also Crowther *et al*., 2016) requires evidence-based understanding of the FIO sources and processes in a catchment. ***Is this evidence available in Scotland and what is the best strategy for identifying the area or zone of influence in BW and SW catchments?***

Addressing these challenges is essential for identifying catchment-based FIO sources and for addressing their impacts on BWPA and SWPA.

1.3 Objectives

In the context of the limitations of regulatory FIO monitoring and the challenges facing blitz monitoring, the objectives of this project as set out in the project's specification are:

1. Review previous work on FIO investigations to identify predominant sources – which monitoring techniques/ equipment have been used successfully to track FIO sources during deployment?
2. Review current technology – which new technologies are likely to be applicable and practical?
3. For the monitoring techniques/options listed below and any others identified by the review, how often would SEPA need to sample to identify the major sources of FIO in a catchment? (Note: accurate source apportionment is not required):
    - o Use of tryptophan "bacti-meter" for field use to follow source strength up a watercourse to find hotspots.
    - o Use of other "proxy" measurements such as conductivity/ ammonia/ turbidity to follow source strength up a catchment to find hotspots.
    - o Use of in-situ continuous measurement (e.g. bacti-meter) at end of catchment to check and verify modelled exports.
    - o Use of on-site filtration of water samples collected during field investigations to reduce carrying/ transport issues and potentially increase preservation times.
4. Identify monitoring strategies depending on the pressure profile of a catchment - e.g. wastewater treatment works (WwTW)-dominated, septic tank-dominated, livestock-dominated, urban/ cross connection-dominated, upland wildlife and sheep dominated. An overall expert judgement or broad guidelines may be required.
5. Visualise the daily/ seasonal flux of FIO concentrations and loads expected from each source type (e.g. septic tanks, roosting birds, livestock, STW discharges to better understand FIO variability). These graphs could be conceptual based on expert judgement if empirical data is unavailable.

1.4 Structure of the report

This report includes the following sections:

- Section 2 describes the research methodology and data used.
- Section 3 outlines the evidence-base on in-stream FIO variability and FIO monitoring technologies and strategies to address objectives 1-5.
- Section 4 summarises project findings for each objective and evidence-based recommendations.
- Section 5 provides concluding remarks.

2.0 Materials and Methods

To address the objectives of this project, we undertook a literature review, evaluated the results, developed a desktop approach to catchment surveys and developed recommendations for a practical monitoring strategy to help identify the area of influence and to detect FIO hotspots and track FIO from different types of sources. Where empirical knowledge (e.g. on FIO monitoring technologies) was limited or not available, recommendations on FIO variability were based on consultations with SEPA staff and experts from the James Hutton Institute (JHI). Knowledge gaps were also flagged up.

Literature review: We undertook a literature review to inform all the objectives of this project. Appendix II.1 summarises the literature review methodology. We gathered evidence referring specifically to types of FIO sources, variability and timing of FIO discharges to watercourses, and to factors influencing FIO variability in-stream. We reviewed the evidence-base on in-stream microbiological monitoring in the context of water quality downgrades in bathing and shellfish waters due to faecal pollution in Scotland and internationally. Applicability of emerging, currently used and proxy FIO monitoring techniques and microbial source tracking technologies specific for detecting each type of source was assessed based on the criteria presented in Figure 1. It must be also noted that a systematic review of the literature on processes influencing FIO pathways within catchments was out with the scope of this project.

Desktop method: This informed Objective 4. Based on the findings of the literature review, we developed a desktop GIS-based approach to help assess place-based risk of in-stream FIO contamination and thereafter prioritise monitoring towards sites posing greatest risk of FIO delivery from catchment to BWPA and SWPA.

Figure 1. Criteria used for assessing microbiological monitoring techniques and technologies (Objectives 1-2) with respect to their applicability in investigating locations and types of FIO sources.

The desktop approach was specifically developed based on advice and practice described in: EPA Victoria 2007; Nanne *et al*., 2011; Kinzelman and Ahmed 2015; US EPA 2010; 2013; Disney *et al*., 2014; Lindberg 2010; Miller and Dorn 2016; Neil *et al*., 2018. Trials focused on two catchments (i.e. Nairn and Loch Ryan), which were selected in consultation with SEPA from an initial list of nine BW catchments and five SW catchments. The data layers and sources are described in Appendix II.2 and summarised in Appendix II-Table II.2a (layers related to FIO sources) and Appendix II-Table II.2b (Ordinary Survey-OS and catchment and protected area boundary data). Appendix II.3 describes the GIS-based method developed here.

3.0 Literature review

3.1 Regulatory background

The regulatory background and the policy drivers for blitz monitoring have been briefly discussed in Section 1.2 and are further detailed in Appendices I.1-I.5. Here we elaborate on the regulatory gaps regarding the control of FIO discharges generated from septic tanks.

3.1.1 Septic Tank Systems (STS): FIO pollution issues

What is known about STS in Scotland?

The number of consented STS in Scotland has been estimated to be 51,700 (Brian McCreadie, pers. com., March 2019), with a review of the exact number and characteristics of consented STS being currently underway. Experts' estimates of the overall numbers vary. Anthony *et al*. (2006), differenced the Ordnance Survey Postcode Address Points register against the number of households charged for drainage services by Scottish Water, and estimated that 184,320 properties were on STS. O'Keeffe *et al*. (2015) reported that more than 161,000 properties in Scotland use STS, of which, only 38% are registered with SEPA. Available evidence also suggests historic problems due to misconnections, i.e. discharges of untreated (also reported as raw or crude) sewage directly from domestic sink, bath, shower, toilet, washing machine, and dishwasher waste into watercourses (e.g. Dudley and May 2007; Withers *et al*., 2011; 2014; see also sanitary survey reports compiled by CEFAS and cited in Akoumianaki *et al*., 2018).

Practically, it is unknown how many STS continue to discharge their effluents directly to rivers and to coastal waters and where soakaways may be posing a faecal pollution risk. There is also limited information on the age and performance characteristics of STS, such as frequency of sludge removal, type and suitability of soakaways, and distance of soakaways from watercourses (Brian McCreadie, SEPA, pers. com. December 2018). SG has provided guidance in the Handbook of Septic Tanks (2010). However, it is unknown whether soakaways are located and designed according to best practice and SG guidance.

Are STS point or diffuse pollution sources of FIO pollution?

Misconnections and STS discharging their effluent directly to surface waters act as point sources (e.g. Richards, *et* *al*., 2016b). However, STS have also been shown to act as diffuse pollution sources to streams and coastal waters through groundwater contamination from soakaways, independent of and in addition to stormwater runoff (Hayes *et al*., 1990; Cahoon *et al*., 2016). STS can act as point sources during baseflow conditions and as diffuse pollution sources during stormflows (Withers *et al*., 2014). STS also qualify as continuous, rainfall-independent, sources of faecal pollution (e.g. Hayes *et al*., 1990; Withers *et al*., 2014; Cahoon *et al*., 2016; Richards *et al*., 2016 a; b).

Are STS regulated as point or diffuse FIO pollution sources?

The CAR framework implies that STS clusters and STS serving more than 15 p.e. are regulated as point sources. In addition, the new general binding rules that came into force on 1 January 2015 by the UK Government stipulate that discharges from septic tanks directly to a surface water are not allowed and that those who have a STS that discharges directly to a surface water will need to replace or upgrade the treatment system by 1 January 2020 (UK Government 2018). However, given the uncertainty about the location and performance of STS in Scotland, STS may also be regulated as diffuse pollution sources in the context of the priority catchment approach.

3.1.2 Regulations related to STS siting and sewage sludge disposal in Scotland

STS siting

Proper siting of an STS is an essential first step in reducing potential pollution issues. It cannot be overstated that regardless of technological advances in rural wastewater treatment, unsuitable STS locations will increase the risk of FIO pollution. Site conditions such as slope, soil type, distance to water table, distance to receiving water are all sensible measures to consider prior to installation of any STS (see review by O'Keeffe *et al*., 2015). Scottish Building Standards (BSI 2008) and the SG Handbook of Septic Tanks (2010) provide some guidance on STS design, with reference to BS 6297. This specifies that an appropriate distance for siting soakaways to minimise the risk of pollution is 50m away from a drinking water source and 10m away, horizontally, from any water course, permeable drain, road or railway. The Handbook of Septic Tanks (2010) also specifies system design requirements and design of soakaways, which are not further discussed here.

FIO in sewage sludge spread as soil amendment

The Sewage Sludge Directive 86/287/EC does not specify limits for *E. coli* counts as a faecal contamination indicator in sewage sludge, but specifies general land use, harvesting, and grazing limits to provide protection against the risk of animal and human infection. However, the revised version of the Sewage Sludge Directive (Working Document 3rd Draft cited in Healy *et al*., 2017), recommends that *E. coli* in sewage sludge must be below or equal to 1 × 103 CFU g^{-1} dry weight (dw) and that the sludge must have limited spores of *Clostridium perfringens* (< 3 × 103 g^{-1} dw) with an absence of *Salmonella spp.* per 50 g wet weight (ww) (Healy *et al*., 2017).

3.2 FIO catchment-based sources

Table 2 summarises the types of catchment-based sources.

Catchment-based sources of FIO pollution in Scotland include (Rees *et al*., 2010; Dulfour and Bartram 2012; Meals *et al*., 2013; Ouattara *et al*., 2014; Ellis and Butler 2015; Cho *et al*., 2016; Pachepsky *et al*., 2017; Neil *et al*., 2019):

- Permitted sources, such as:
    - the Granted Point Sources (GraPS) under CAR (Appendix I.1) and registered STS in Scotland; CSO and stormtank overflows (STO) deliver to watercourses a mixture of untreated wastewater and surface runoff water containing FIO of both human and animal origin.
    - Land managed with manure or sewage sludge under the General Binding Rules (GBR) (Appendix I.1), which may deliver FIO of human and animal origin.
- Residential sources, such as: failing or inadequately built STS; illicit sewage discharges from industry or households (i.e. misconnections); broken sewerage network; cross-connections (i.e. diversion of stormwater into the wastewater network, or wastewater into the stormwater system), and stormwater runoff in rural and urban areas. These sources may deliver a varying combination of FIO derived from human, livestock, wildlife and pet sources.
- Livestock, in the form of deposition of faeces on pasture land and directly in-stream as well as in the form of manure/slurry storage and farmland application. Livestock FIO may be derived from: cattle (dairy/beef); horses, swine, sheep and goats, and poultry.
- Wildlife, in the form of faeces deposited in breeding sites, rural or urban resting sites and directly in-stream.
- Transit sources, such as reservoirs of FIO in soils, riparian zones, stream bed and estuarine sediments, suspended matter, and shallow groundwater.

Table 2. Classification of sewage effluent by type of activity. Source: Environment Agency, 2018; CAR, 2018.

Type of effluent	Type of premises	Activity generating sewage containing effluent (Typical examples)	Source of microorganisms
Domestic	• Single household • Small scale* restaurants, hotels/campsites. hospitals	• Toilet flushing • Personal washing • Cooking • Washing dishes and cooking equipment • Household cleaning (using detergents)	Predominantly human
Trade	• Residential • Commercial • Industrial	• (In addition to domestic effluent) • Chemical toilets • Launderettes • Food processing plant • Swimming pools • Large hospitals • Industrial processes • Intensive livestock installations	Human/animal
Organic	• Commercial • Industrial	• Domestic sewage • Freshwater fish farm or hatchery • Intensive livestock installations	Human/animal
Other	Landfill	• Landfill leachate production	Human/animal

* Comparable to household scale.

Except for exclusively domestic sewage effluent from WwTW and community septic tanks, the contribution of human and animal-derived FIO in the trade, organic or other type of effluent from GraPS is expected to be specific to the activity generating wastewater (Table 2).

3.3 FIO transport pathways across catchments

FIO may enter bathing and shellfish waters through direct, point source and indirect, diffuse source pathways:

- Direct pathways from point-sources involve discharges from discrete conveyances such as pipe and drain outflows to surface waters such as streams, rivers, lochs and coastal waters, and stream discharges draining sub-catchments, such as SEPA's waterbody catchments, to the river network and the coast (Rees *et al*., 2010; Meals *et al*., 2013).
- Indirect pathways from diffuse pollution sources are determined by hydrological and landscape connectivity (e.g. Neil *et al*., 2019) and include discharges via:
    - i. Urban and rural land runoff (Rees *et al*., 2010; Meals *et al*., 2013)
    - ii. Groundwater infiltration to coastal waters (Boehm *et al*., 2004; Izbicki *et al*., 2012; Russell *et al*., 2013; Sieyes *et al*., 2016)
    - iii. Shallow groundwater seepage zones in the streambed during baseflow (Collins and Rutherford 2004);
    - iv. Resuspension of sediments deposited in the streambed, lakebed, intertidal areas and the seabed (Wu *et al*., 2009; Kim *et al*., 2010; 2015; Wyness *et al*., 2018).

FIO entrained in effluent, runoff and steam-water are transported in suspension as single cells, clumped cells, or, in the case of runoff and stream water, as cells attached to manure or soil particulates (Tyrrel and Quinton 2003; Muirhead *et al*., 2005). FIO bound in suspended particles within streams can settle out of the water column as water velocity declines. This leads to their accumulation in streambed and coastal sediments (Cho *et al*., 2016), where they form part of the legacy FIO at a site (Pandey *et al*., 2014).

It must also be noted that that temporal or permanent water saturation of the shallow aquifer allows FIO to move quite far along preferential pathways in subsurface soils (Jamieson *et al*., 2002; Ferguson *et al*., 2003; Tyrrel and Quinton, 2003), posing a risk of contamination to groundwater resources.

3.4 Timing of FIO discharges

FIO discharges may be (Rees *et al*., 2010; Kay *et al*., 2008a; b; c; Kay *et al*., 2010; Meals *et al*., 2013; Campos *et al*., 2013; Cahoon *et al*., 2016; Richards *et al*., 2017; EEA 2009; Muirhead *et al*., 2011; 2018; Pachepsky *et al*., 2017;2018; Cho *et al*., 2016):

- Rainfall-independent discharges, which refer to discharges from both point- and diffuse pollution sources regardless of flow conditions. Variation in these discharges may be diurnal, seasonal, or event-scale. Emergency discharges, which occur rarely due to poor maintenance or malfunctioning of the sewerage network, are rainfall-independent discharges.
- Rainfall-dependent discharges, which refer to point-source and rural or urban diffuse pollution discharges triggered by surface runoff during rainfall or irrigation.

A classification of the types of FIO sources by the timing of discharges to the water environment (Figure 2) shows that FIO discharges from diffuse pollution sources such as farmland, wildlife breeding and resting sites as well as sediments can be both rainfall-dependent and rainfall independent. Understanding timing of discharges is key to identifying reliably background in-stream FIO concentrations (e.g. during baseflow) and capturing the relative contributions from different sources at different times and during stormflow.

Additional factors influencing the timing of FIO discharges are summarised below.

- The type of sewer system (e.g. combined or separate) determines how and whether additional factors play a role, such as catchment characteristics, extent of impermeable/built-up areas, and potentially operational management of the treatment system (Kay *et al*., 2008a; Kistemann *et al*., 2008).
- The observed flow of the treated effluent at the outfall of centralised WwTW is normally continuous and tends to have a repetitive diurnal pattern determined by the cycle of WwTW operation, the size of population served, the length of sewerage network and the storage capacity of the treated effluent, although it is still subject to variability (Pescod ,1992; Lenart-Boron *et al*., 2016, Butler and Graham, 1995).
- Small centralised WwTW (maximum 5000 p.e.) may display greater diurnal variations in sewage loading and flow resulting from sewage over- or under-loading of the treatment plant and fluctuations in the

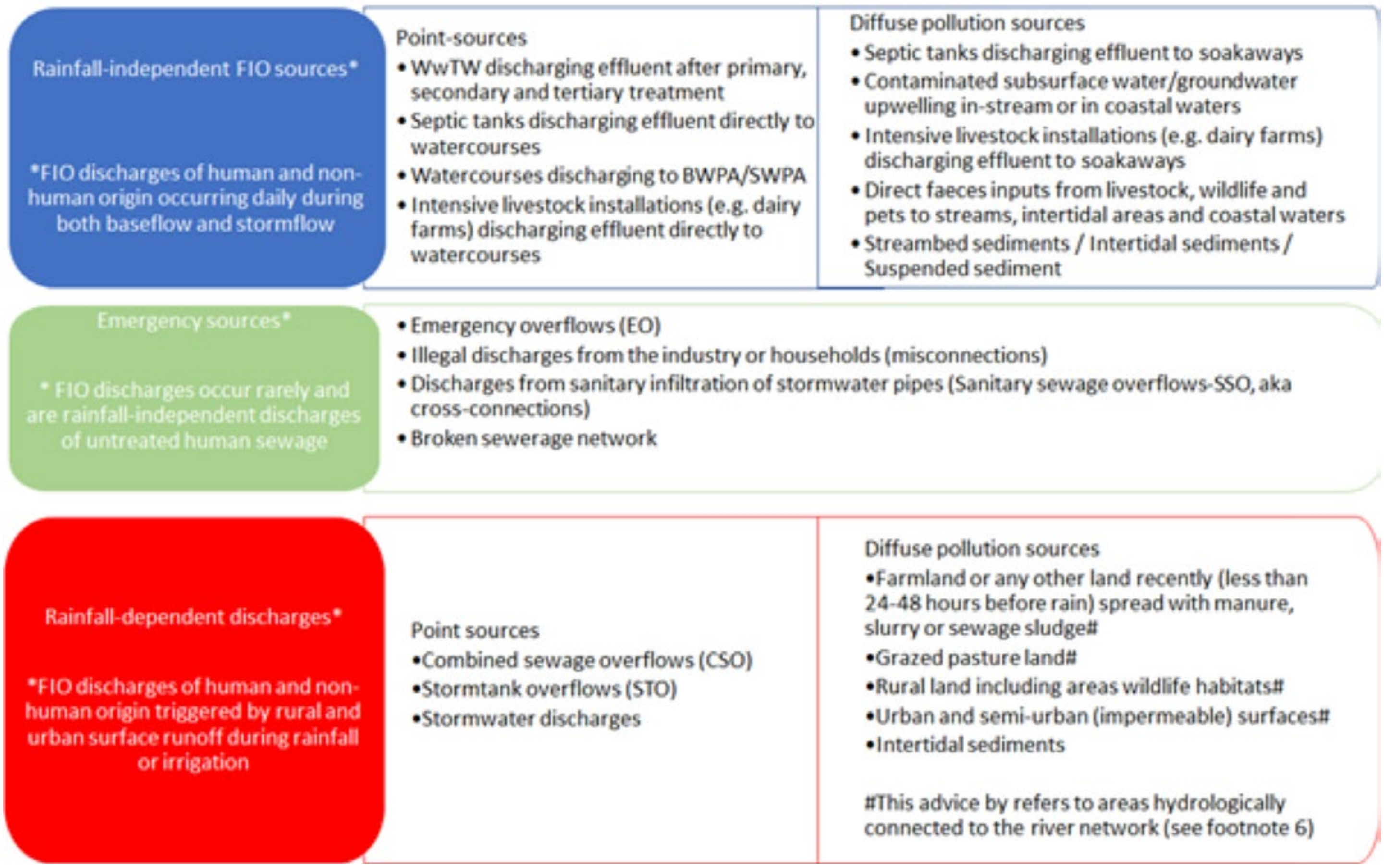

Figure 2. Potential catchment-based sources of faecal pollution in relation to timing of FIO discharges to the water environment. Sources: DEFRA 2009; Rees *et al*., 2010; Meals *et al*., 2013; Campos *et al*., 2013; Cahoon *et al*., 2016; Richards *et al*., 2017; EEA, 2009; Kay *et al*., 2010).

influent quality (ISISQ, SMAS SINTRA and QUESTOR 2006).

- Private domestic septic tank effluent discharges may show a higher level of variability than centralised treatment works due to variations in household water use and wastewater and effluent production on a local scale (O'Keeffe *et al*., 2015).
- Diurnal variability of FIO in septic tank effluent and the rate of their discharges to soakaways is specific to each household and depends on the: diurnal water use pattern; type of indoor use (i.e., laundry, toilet, shower, etc); the use of chemicals (such as laundry products); retention time in the septic tank (i.e. duration of primary treatment); and the presence of misconnections (see Section 3.1.1.1) of roof drains or surface water drains to a septic tank (Lucas *et al*., 2017; O'Keeffe *et al*., 2015).
- The frequency of rain-dependent overflows is site- and year- specific and depends on the size of the public sewer network; the area and imperviousness of washing surfaces; climate and weather, especially rain event frequency and duration; and the number of dry days before any rainfall event (Puerta and Suarez 2002; Barco *et al*., 2008; Galfi *et al*., 2016).
- CSO and STO discharges are temporally variably (Kay *et al*., 2008a) and are generally influenced by seasonal changes in human population due to tourism (Campos *et al*., 2013).
- Septic tank effluent discharges may show diurnal and seasonal variability reflecting peak demands in the early morning and late evening and contribute consistently under varying flow conditions, having greatest impact on in-stream nutrient concentrations under low-flow conditions (Palmer-Felgate *et al*., 2008).
- The form of spreading manure and slurry can also affect FIO survival in the soil and thereafter influence FIO load in farmland runoff. Hodgson *et al.* (2016) observed a significant increase in FIO persistence (measured as half-life of E. coli and IE) when slurry was applied to grassland via shallow injection and significantly higher decay rates for broadcast application of slurry to the soil surface when temperature and UV were highest. However, in-stream FIO pollution risk from slurry spread via shallow injection also depends on site-specific factors such as the presence and depth of tile drains, the soil type and the presence of, for example, preferential pathways such as soil macropores (Hodgson *et al*., 2016).

3.5 FIO discharges: composition and load

Wastewater and stormwater discharges

- FIO load in effluent from WwTW is determined by type of source; level of treatment; population equivalent (p.e.); volume of discharge; system performance; age and maintenance of sewage transport infrastructure; type of the sewerage system (i.e. combined versus separated stormwater and domestic wastewater) (Touron *et al*., 2007; Kay *et al*., 2008a;b; Campos *et al*., 2013a; Kistemann *et al*., 2008; Richards *et al*. 2017; Lusk *et al*., 2017).
- FIO in stormwater runoff is derived from roof and road surfaces as well as from cross-connections to urban sewerage systems and thus it may be of avian (e.g. urban gulls), wild animal and human origin (Kelsey *et al*., 2004; O'Keefe *et al*., 2005; Sauvé *et al*., 2012; Parker *et al*., 2010; Pandey *et al*., 2014; Dufour *et al*., 2012).

Effectiveness of wastewater treatment

In general, the FIO removal efficiency during treatment is mainly dependent on the type of treatment (primary, secondary, or tertiary) and FIO in the influent, which in turn is determined by the size of population generating wastewater. The type of sewage system (e.g. STS, combined or separate sewer system) determines how and whether additional factors play an important role. For example: catchment characteristics and rainfall patterns influence the composition and FIO load of CSO discharges; soil types influence the composition of leachate from STS soakaways; and potentially operational management of the treatment system influence discharges from WwTW (Kay *et al*., 2008a; Kistemann *et al*., 2008). FIO concentrations in the effluent have been found to vary between systems providing the same level of treatment and to be related to temperature variations, natural UV-disinfection, disinfection procedures, and filter design (Robertson *et al*., 2006; Kay *et al*., 2008; Kistemann *et al*., 2008). Additional factors such as age and capacity of the treatment plant and external factors influence FIO and pathogen removal efficiencies of WwTW and therefore must be assessed individually in order to evaluate a particular treatment type of treatment for a certain microbiological parameter (Kistemann *et al*., 2008).

Scotland – FIO in effluent

Knowledge of the likely FIO concentrations produced by different sewage treatment interventions is essential for the design of monitoring to detect FIO sources (US EPA 2010; Lindberg *et al*., 2010; Reischer *et al*., 2011; Nnane *et al*., 2011; Kay *et al*., 2008a). However, our knowledge of FIO in the effluent from different types of GraPS in Scotland is limited (see Kay *et al*., 2008a). It must be also noted that data on population served or discharge volume of the GraPS in the trial catchments (i.e. Nairn and Loch Ryan) were unavailable to the project team. To provide evidence analogous to the Scottish context, we took account of the size of the selected trial catchments i.e. Nairn (catchment area-c.a.: 338.5 km^2) and Loch Ryan (c.a.=132.5 km^2); the population density in Scotland, which was found to range from 66 to 68 persons/km^2 since 1961; and the population equivalent (p.e.) in Nairn and Loch Ryan based on 2011 Census population data[5].

In this context, we looked up FIO discharge data from point sources serving populations in river catchments smaller than 350km^2, serving a p.e. smaller than 50,000 and areas of low population density. Our findings are presented in Table 3.

The evidence presented in Table 3 should not be considered definitive because it is based on limited empirical data from Scotland and few other analogous areas and because it shows an inherent variability in FIO in treated effluent.

This evidence also suggests that FIO discharges from different types of point sources, can be ranked as follows:

1. High FIO discharges are associated to untreated wastewater from EO, broken sewerage network and direct discharges (misconnections) of wastewater from small communities or any other public activity (e.g. businesses) as well as from individual households.

5 The results for p.e. were: Nairn and Cawdor (Nairn BW Catchment) =13,041 based on Scotland's Census (2011) and for Loch Ryan SW catchment=13,258 based on calculations by Akoumianaki et al. (2018).

Table 3 . FIO concentrations in sewage discharges categorised by type or treatment of wastewater. IE: Intestinal Enterococci; CV=Coefficient of variation, i.e. coefficient of variation (CV) is defined as the ratio of the standard deviation to the mean. * this refers to a range of flow conditions including both baseflow and stormflow.

Type of treatment	IE (CFU/ 100ml) (Mean)	*E. coli* (CFU/ 100ml) (Mean ± CV)*	References
Untreated crude sewage effluent	Mean – baseflow: 1.9 × 10^6 Mean – stormflow: 8.9 × 10^5		Kay *et al*., 2008a[1]
Untreated sewage from CSO/STO	Mean: 3.8 × 10^5		Kay *et al*., 2008a
WwTW: Primary settled sewage	Mean – baseflow: 2.4 × 10^6 Mean – stormflow : 1.9 × 10^6		Kay *et al*., 2008a
Septic Tanks: **Primary treatment**	Mean – baseflow: 9.3 × 10^5 Mean – stormflow: 4.3× 10^5		Kay *et al*., 2008a
		10^5 – 10^8	Lusk *et al*., 2017
		5.0 x 10^5	Gill *et al*., 2007
WwTW Secondary treatment (without trickling filter)		1.6 X 10^4±0.3 – 8.5 X 10^4±1.3	Kistemann *et al*., 2008[2]
WwTW: Secondary treatment (with trickling filter)	Mean – baseflow: 4.1 × 10^4 Mean -stormflow: 5.7× 10^4	0.6 X 10^4±1.2 – 0.4 X 10^4±0.8	Kistemann *et al*., 2008
WwTW: Sand filtration		0.4 X 10^4±1.5 - 0.8 X 10^4±1.1	Kistemann *et al*., 2008

1 Data came from 11 UK study catchments, only one (i.e. Irvine) located in Scotland. 162 sewage-related discharge sites were monitored, typically over a 2–3-month period during a single summer bathing season (May–September) between 1993 and 2005, with "opportunistic" sampling during stormflows. For each sampling point, a 'typical' dry weather flow pattern over a 24-h period was derived by taking the average hourly flow values for each hourly time-step from at least 7 days of data when flows were unaffected by rainfall. Where the actual flows were observed to be elevated compared with the typical flow pattern at times associated with rainfall, these were categorised as high flows. All storm sewage overflow samples (i.e. STO and CSO) were categorised as high flow.

2 This study by Kistemann *et al*. (2008) was undertaken in the federal states of North-Rhine Westphalia (NRW) and Rhineland Palatinate (RP) in an area that covers the 289 km2 catchment area of the river Swist (length:43.6 km). The population of the investigated area averaged 91,700 in 2001/2002; population density of 322 people/km. The sewage treatment plants drained small sub-catchments (0.2-12km^2) serving 422 to 37,655 p.e. Samples were collected from Autumn to Summer with a total 5-10 replicates of the influent and the effluent.

2. During stormflow, high FIO discharges come from CSO and STO.

3. During baseflow, high FIO discharges are associated to sources discharging effluent after primary treatment, because this type of treatment has the lowest FIO removal efficiency (e.g. Kay *et al*., 2008a). Examples of this type of point sources are public septic tanks, and compact treatment systems providing physical treatment such as sedimentation of solid waste.

4. During baseflow, high FIO discharges may also be associated to the presence of WwTW or STS providing secondary treatment, but this depends on local circumstances such as p.e. and type of secondary treatment, with sand filtration reducing considerably risk to public health.

5. Low FIO discharges are associated to the presence of WwTW and STS performing tertiary treatment.

Scotland – FIO from land use/land cover.

FIO export is higher from catchments dominated (i.e. more than 75% of catchment area) by pasture land and urban areas than in catchments dominated by arable land or forests, and during stormflow than during baseflow (Kay *et al*., 2008b, McDowell 2008).

Kay *et al*. (2008b) estimated FIO export coefficients per type of land use under base-flow and high-flow conditions from in-stream FIO concentrations and discharge estimates collected from 205 catchments in the UK; of these, 52 were in Scotland. This data reflects the combined point- and diffuse-source inputs to the watercourses monitored. Based on available land cover and land use data, Kay *et al*. (2008a) calculated the FIO export coefficients for catchments covered by more than 75% by any of the following types of land use/land cover: improved grassland (IP), rough grassland (RG), woodland (WL) as well as for rural, semi-urban and urban land use. The estimated export coefficients per type of land use are presented in Appendix III in Table III.1.

The study by Kay *et al.* (2008a) showed:

(i) Higher FIO export coefficients from catchments dominated by semi-urban and urban areas compared to catchments dominated by rural and use.

(ii) Higher FIO export from catchments dominated by improved grassland compared to catchments dominated by rough grassland and woodland.

(iii) Increase in FIO export during high flow conditions by one to two orders of magnitude even from rough grassland and woodland.

3.6 FIO pollution risk

Risk from rainfall-dependent point sources of FIO pollution

- FIO from CSO and STO contribute a considerable but interannually variable proportion of the total FIO flux to watercourses during storm events (Kay *et al*., 2010).
- CSO discharges (sewage diluted in stormwater) typically peak before river flows peak within a couple of hours after the beginning of rainfall and can induce very rapid and important increases in the concentration of FIO (up to two orders of magnitude) and pathogens in the adjacent receiving waters (Passerat *et al*., 2011; Ouattara *et al*., 2014; Madoux-Humery *et al*., 2016).
- STO discharges at WwTW occur after river flows peak. Here, it is useful to remember that STO are normally required to delay the polluting "first flush" from CSO at a time when flows in the receiving river, BWPA or SWPA, will not have responded to rainfall as quickly as the impermeable areas served by the combined sewer (SEPA 2014).
- FIO concentrations in the stormwater component of CSO discharges increase with population density, percentage of impermeable cover (i.e. built-up areas), density of housing, domestic pet activity, temperature, and, in estuarine areas, tidal stage (Kelsey *et al*., 2004; Parker *et al*., 2010).
- In rivers, risk of FIO pollution can be determined by: the ratio between the CSO discharge rate and the river flow rate, which determines dilution; vertical mixing conditions; and the concentration of microorganism in the CSO (Deeks *et al*., 2005; Rechenburg *et al*., 2006; Olds *et al*., 2018; Kay *et al*., 2008a).

Risk from rainfall-independent point sources of FIO pollution

FIO concentrations in the effluent from WwTW vary between systems providing the same level of treatment as a result of variations in temperature, disinfection procedures (e.g. application of natural UV-disinfection or not), treatment design and age and capacity of the treatment plant (Kay *et al*., 2008a; Kistemann *et al*., 2008). Therefore, individual studies are key to understanding site-specific WwTW impact and FIO pollution risk (Kistemann *et al*., 2008).

Risk from septic tanks

Diffuse pollution risk of surface waters and groundwater from STS increases with:

- High FIO in the effluent over spilled to soakaways (Lusk *et al*., 2017).
- Generally, proximity of STS to surface waters, and/or fractured bedrock (e.g., Katz *et al*., 2010; Withers, *et al*., 2014; Lusk *et al*., 2017). For example, in Scotland the Handbook of Septic Tanks (2010) specifies that soakaways should be located at a distance greater than 10m from watercourses.
- Proximity of STS soakaways to watercourses, especially at areas where soils have high soil leaching potential and therefore afford little chance for retention in the soil (Lusk *et al*., 2017; Stevik *et al*., 2004). Under these circumstances, FIO effluent once over spilled to the soakaway may migrate downward through the vadose zone (i.e. shallow, unsaturated groundwater) and into groundwater, and eventually infiltrate into surface waters through the stream bed or seabed when the water table is elevated.
- Proximity of STS soakaways to watercourses at areas where soils have limited capacity to store water (Lusk *et al*., 2017; Stevik *et al*., 2004; US EPA 2010), whereby STS effluent over spilled to soakaways can reach waterbodies through surface and subsurface runoff.
- High housing densities in rural areas. For example, a density greater than 40 domestic STS per square mile is considered as high risk in terms of FIO load in an area (Katz *et al*., 2010; Borchardt *et al*., 2003). Further, Cahoon *et al*. (2006) showed that improperly performing STS located at a high density" of 20/ha (i.e. or 2000/km^2) in permeable soils adjacent to estuarine waters can be more important contributors to faecal pollution of shellfish waters than stormwater runoff. Yates *et al*. (1985) report that USEPA recommend a maximum density of 1 STS per 16 acres, 15 STS /km^2.
- Unsuitable tank size with respect to number of individuals served (Richards *et al*., 2016a).

Individual domestic private STS are usually associated with small volumes of domestic sewage discharge but malfunctioning or poorly sited septic systems may be locally significant as sources of *E. coli* and Norovirus to coastal waters (Campos *et al*., 2013; Campos and Lees 2014). The degree of 'purification' that occurs during primary treatment in the septic tank will determine FIO load in the final effluent to the soakaway. Purification is influenced by: retention time[6] within the tank; seasonality of usage; use of chemicals; temperature (e.g. higher temperature reduces pathogen survival within the septic tank); and turbidity/suspended solids within the tank[7] (O'Keefe *et al*., 2015). Given that these factors remain poorly reported, it is difficult to calculate with precision the contribution of septic tank effluent to total microbial loads within a waterbody. According to rough estimates, 23.5% of the diffuse source *E. coli* load or 7.6% of the total load (diffuse and point source) to Scottish groundwaters and surface waters may result from private STS discharges (SNIFFER 2006 cited on O'Keefe *et al*., 2014).

Risk from livestock farming

BWPA and SWPA downstream of pasture land are at increased risk from faecal contamination (Campos *et al*., 2013). Results of sensitivity analyses of modelled output accounting for the effect of direct inputs and dairy farm effluent on in-stream FIO from previous studies (Collins and Rutherford 2004; Muirhead *et al*., 2011; Wilcock *et al*., 2006) are summarised below.

- In-stream: FIO concentrations are sensitive to *E. coli* concentration in the source material (e.g. faeces, dairy farm effluent); the number of days per year dairy farm effluent was generated; the number of days animals have to walk through the stream to access food; the relative contributions of livestock and wildlife faeces directly deposited in the stream; and stream flow rate. These findings show the need for good input data sets describing livestock and wildlife distribution, *E. coli* in faecal material, streamflow, and reliable modelled data.
- At the farm-scale: sporadic livestock and wildlife access to stream networks will have little effect on the median FIO concentration but will increase the maxima concentrations. However, the effect of sporadic wildlife access cannot be addressed with FIO control measures.
- At a catchment scale: the multiple farm inputs will tend to average out, but the variability and sporadic nature of the farm inputs are likely to be a key cause of the variability in FIO concentrations measured in agricultural catchments during baseflow.

Risk from diffuse sources of FIO pollution

- Risk of diffuse faecal pollution in-stream and in BWPA/SWPA increases with (e.g. Oliver *et al*., 2009 a;b; Kay *et al*., 2012; Murphy *et al*., 2015a;b; Porter *et al*., 2017; Chadwick *et al*., 2008; Collins and Rutherford 2004; Muirhead *et al*., 2011; Wilcock *et al*., 2007):
    - o Density of grazing livestock, wildlife and pets

6 Retention time depends on the size and type of the septic tank; its maintenance (e.g. how often it is emptied to enable sufficient physical purification); and the presence of misconnections (Lusk et al., 2017; Withers et al., 2014). The misconnection of roof drains or surface water drains to a STS can increase hydraulic loading and, during storm events, it could increase turbulence and reduce effluent retention time in the STS (O'Keeffe et al., 2014).

7 Adsorption varies by FIO and pathogen species.

- o Density of private STS
- o Faster surface and subsurface flowpaths/higher landscape or hydrological connectivity[8]
- o Slope
- o Limited slurry storage capacity in source-catchments and implementation of manure treatment technologies
- o Low uptake of agri-environment FIO control measures e.g. wetlands, and buffer strips.

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8 Landscape connectivity can generally be defined as the probability that a certain point in the landscape is capable of transmitting material to another point. For a point to be considered hydrologically connected, it must be generating runoff and transmitting the flow vertically downwards and downslope to the stream/river channel and thereof downstream (Brierley et al., 2006). Structural connectivity is defined as the extent to which landscape units (at multiple spatial scales) are physically linked to one another; and functional connectivity, is defined as the way in which interactions between multiple structural characteristics within a catchment affect geomorphological, ecological and hydrological processes (Wainwright et al., 2011). For a comprehensive review of the concept of "connectivity" in relation to catchment modelling and measuring to understand connectivity readers are advised to refer to Keesstra et al., 2018.

- Pet and livestock wastes can be an important source of faecal contamination and a number of pathogens (e.g. *Giardia* sp., *Cryptosporidium parvum*, and *Salmonella* sp.), especially in built-up areas (Meals *et al*., 2013). For example, E.coli production rates from dogs, cats, cattle and sheep are greater than rates from geese and pigeons by an order of magnitude (Table 4).
- Wildlife, both mammals (e.g. deer, foxes, rabbits, rats and other local species) and birds, can act as pathogen reservoirs especially when they reach high density close to coastal waters (e.g. shoreline or small streams directly discharging to coastal waters), whereby there is little opportunity for FIO die-off (Meals *et al*., 2013); see Table 4.

3.7 FIO variability in-stream and in bathing and shellfish waters

General information

- Multiple sources influence FIO concentrations in-stream and their relative importance changes temporally and varies with land use and type of FIO growing within the sewerage network (Gourmelon

Table 4. Typical FIO concentrations and excreation rates of various animals living in coastal and freshwater environments. Key: EC=*E. coli*; FS=Faecal streptococci; FC=Faecal coliforms.

Animal	Density (no./g)	Excretion rate (g/day)	Load (/day)	EC production rate (cfu/day/head)	Reference
Coastal bird	EC=7.2X10^7 FS=1.6X10^8				Leeming *et al*., 1998*
Ducks	FC=3.3X10^7 FS=5.4X10^7	68 68	FC=2.2X10^9 FS=3.7X10^9		CWP 1999*
Gulls			EC=2X 10^9		Whither *et al*., 2005*
Waterfowl	FC=3.3X10^7	120	FC=4.0X10^9		CWP 1999*
Humans				1X10^9	Metcalf and Eddy 1991#
Dogs / cats				2.5X10^9	Horsley and Witten 1996#
Cattle				2.7X10^9	Metcalf and Eddy 1991
Hogs				4.5X10^9	Metcalf and Eddy 1991#
Sheep/Goats				9X10^9	Metcalf and Eddy 1991#
Poultry				1.3X10^8	Metcalf and Eddy 1991#
Horses				2.1X10^8	ASAE 1998#
Deer				1.8X10^8	Zeckoski *et al*., 2005#
Geese				1X10^7	Alderisio and DeLuca 1999#
Ducks				5.5X10^9	Metcalf and Eddy 1991#
Raccoons				5.7X10^7	Yagow 1999#
Pigeons				8X10^7	Oshiro and Fujioka 1995#

*Cited in McCarthy *et al*., 2009; #Cited in Lefevre *et al*., 2014. Literature sources provide FC production rates, which were converted to *E. coli* by applying a conversion factor of 0.5 based on Doyle and Erikson (2006 cited in Lefevre *et al*., 2014). Therefore, *E. coli* production rate=0.5X FC production rate.

et al., 2010; Durfour *et al*., 2012; Garcia-Aljaro *et al*., 2019). For example, FIO concentrations in small coastal streams are influenced by non-human (e.g. wildlife) sources during dry seasons and from human sources during normal rainfall periods (Shehane *et al*., 2005). Further, dominant FIO sources in streams draining upland catchments have been found to be ruminant livestock and wildlife in different degrees varying between and within storm events (Reischer *et al*., 2011).

- In rural catchments with mixed sources originating from livestock and humans, presumably from septic tank misconnections and STS, in-stream nutrient concentrations show diurnal patterning during baseflow (Murphy 2015; USEPA 2010). However, in-stream FIO diurnal variability remains poorly studied and there is limited evidence on how solar radiation interacts with peak human activity (USEPA 2010).
- FIO concentrations in-stream increase before river flows peak when loading to the stream due to wash-off from surface soils is high and streams are turbulent, promoting resuspension of sediment-bound FIO (Baxter-Potter and Guilliland 1988; Jamieson *et al*., 2005a;b; Haack *et al*., 2003; Wyness *et al*., 2019). Subsequently, FIO may fall sharply immediately after peak flow potentially due to depletion of FIO in soil and in streambank and streambed sediments (e.g. Whitman *et al*., 2006).
- Targeted monitoring has revealed that FIO concentrations in bathing waters exhibit diurnal variability consistent with solar radiation effect on any given bathing day and that the variation can span across different bathing water classifications, with lowest concentrations in the late morning/early afternoon (Wyer *et al*., 2018).
- FIO non-compliances at bathing waters are usually associated with transport of FIO from sources based on land to watercourses through runoff during rainfall and faster transport through the river network during stormflows (Kistemann *et al*., 2002; Kay *et al*., 2010).
- Spatial variability along stream (upstream – downstream) is greater than within stream-reach variability (US EPA 2010). Stocker *et al*. (2018) studied *E. coli* concentration changes in the stream-bottom sediment after high-flow events and reported that growth of the *E. coli* population was pronounced at the stream-reach scale but could be easily missed with the monitoring at the "single- sample" scale.
- Interannual variability in in-stream FIO concentrations in-stream is less studied than event-scale variability but it may vary in relation to change in land use, population and weather/climate (e.g. Chigbu *et al*., 2004; Laurent and Mazumder 2014; Leight *et al*., 2016).

Flow

- The relationship between in-stream FIO and stormflow is not simple. It is determined by a complex combination of factors such as temperature, hydrological connectivity, livestock management practices, wildlife activity, load and age of faecal-reservoirs in-stream and in soils, dilution during stormflow or at the confluence of streams with the main stem of rivers, and channel and bank storage (Baxter-Potter and Gilliland 1988; Jamieson *et al*., 2005a; b; Thomas *et al*., 2007; Neil *et al*., 2018; 2019; Deek *et al*., 2005; Kay *et al*., 2008a;b).
- Diurnal FIO fluctuations in-stream can be more pronounced in slower-flowing, less shaded stream reaches than on smaller, faster and more shaded ones (Traister and Anisfield 2006).
- A potential anthropogenic cause for diurnal FIO fluctuations in-stream is the variable loading of surface waters of raw (untreated) (Bordallo 2003) and treated sewage (Lenart-Boron *et al*., 2016).
- Rise in FIO during stormflow conditions is often attributed to increased hydrological connectivity between catchment-based faecal sources and the river network, mainly through surface runoff (Dwivedi *et al*., 2016; Kay *et al*., 2008b; Tyrrel and Quinton, 2003).
- The lag time between the beginning of rainfall events and sharp rises in FIO concentrations varies from event to event and within events but, generally, small, flashy streams may exhibit shorter lag periods (e.g. Dorner *et al*., 2007).
- The time required for FIO concentrations to recede to pre-storm levels is highly variable among catchments and even for a given catchment (USEPA 2010; Dufour *et al*., 2012).
- In Scotland, FIO concentrations in streams draining rural catchments and downstream of STS are not clearly associated with flow or season (Richards *et al.*, 2017; Neil *et al.*, 2018). However, a CREW study by Akoumianaki *et al.* (2018) showed that shellfish E coli concentrations increase with the level of rainfall at the SW catchments.

Temporal patterns

- Within minutes variability is very small and is most likely to be obscured by event-scale, diurnal and monthly/seasonal variability.
- The evidence base on diurnal FIO variations is poor and where it occurs, it may be overshadowed by event-scale variability. Diurnal variability may be caused by (USEPA, 2010):

(i) The solar radiation cycle, leading to FIO increasing during night and until early morning.

(ii) Peak use patterns, leading to FIO increasing in the late morning and late afternoon/early evening.

(iii) The operational cycle of WwTW.

- Event-scale variability (i.e. rainfall or emergency discharges) is a key cause of FIO temporal variability, with large changes of FIO concentrations being observed during a single event (e.g. USEPA, 2010; Jordan and Cassidy, 2011; Kay *et al.*, 2010).
- Monthly/seasonal/interannual FIO variability is site-specific and may be smaller than event-scale and diurnal variability (USEPA, 2010).

3.8 FIO survival in the environment

Knowing *E. coli* survival rates is important for assessing the severity of contamination that has occurred and making appropriate management decisions (Blaustein *et al.*, 2013). The length of microbial survival in sewage, soils, stream water, and sea water varies widely (Feachem *et al.*, 1983; Park *et al.*, 2016; Ashekuzzaman et al., 2018). Microbial survival also depends on sample storage length (see Section 3.6). The factors influencing FIO survival in the environment are summarised below.

FIO survival in soil

- A risk assessment and uncertainty analysis based on published evidence on *E. coli* decay rates in soils suggested that the residual concentrations in dairy slurry/sewage sludge-amended soil after 20 days would be 45–57% lower than that of the background (not-amended) soil *E. coli* concentration (Ashekuzzaman, et al., 2018), indicating that knowing the timing of farming practices is key to understanding potentially high FIO concentrations in-stream.
- Temperature is the leading environmental variable affecting survival of manure-borne coliforms in soils, with shorter FIO survival times associated with increases in temperature (Sobsey *et al.*, 2006; Frantz *et al.*, 2014; Park *et al.*, 2016) and repeated freeze-thaw cycles (Habteselassie *et al.*, 2008; Natvig *et al.*, 2002; Neil *et al.*, 2019).
- Typically, retention of microorganisms by soil surfaces in soakaways is higher than pathogen die-off, such that irreversible retention of microorganism on soil particles is viewed as the most effective means of protecting underlying groundwater resources from septic system-derived pathogens (Bradford and Harvey, 2016; Schijven and Hassanizadeh 2000).
- In unsaturated soils with high clay and organic matter content, microorganisms in septic tank effluent over spilled to a soakaway may be almost completely removed after percolation through a relatively short vertical distance (e.g. 20-60 cm to the water table) (Lusk *et al.*, 2017 and Stevik *et al.*, 2004), thereby substantially reducing the risk of FIO contamination from STS to groundwater as well as to surface water through groundwater infiltration to streambed.
- In saturated coarse soils with low clay and organic matter content, microorganisms can migrate longer vertical and horizontal distances (Lusk *et al.*, 2017 and Stevik *et al.*, 2004), thereby increasing risk of FIO groundwater and surface water contamination from STS.
- The evidence-base on FIO persistence patterns delivered to soils through manure and slurry applications is limited. For example, Hodgson *et al.* (2016) studied FIO persistence in slurry-amended soils in Devon, UK and found that E. coli in the soil spread with slurry via shallow injection and broadcast application persisted for 131 and 102 days after application, respectively.

FIO survival in streambed sediments

- FIO die-off in streambed sediments has been found to be substantially slower than in water, which can possibly be related to: the presence of mineral particles (Gerba and McLeod 1976; Desmarais *et al.*, 2002); and to less active predation in sediments as well as the reduced threat of UV light (Evanson and Ambrose 2006).

FIO survival in environmental waters

Several models have been developed to describe the key factors influencing FIO survival rates in water (see review by Cho *et al.*, 2016 and literature cited therein). The application of different models to fit E. coli inactivation data showed different stages of survival with distinctly different die-off rates. The linear semi-logarithmic inactivation (LSL) model which was satisfactory only in about 25% of a large E. coli database in waters (Cho *et al.*, 2016), performing better in groundwater, rivers, wastewater and marine waters (Stocker *et al.*, 2014). The Weibull model has been proposed as the superior model to predict FIO inactivation in waters (Cho *et al.*, 2016), although it has been found to perform better in pristine waters (Stocker *et al.*, 2014). Given these uncertainties, we decided to review the factors influencing FIO survival and not survival rates in stream water.

The factors influencing FIO survival (based on the review by Cho *et al.*, 2016 unless otherwise stated) are outlined below.

- Solar radiation. This is recognised as the most influential factor in promoting FIO die-off in the water column. The UVA range of wavelengths (i.e. range from 320 through to 400 nm), is considered to be the most bactericidal, with a UVA radiation level of 60e70 W m^2 being sufficient to reduce the T90 for E. coli to a fraction of a day in sea water despite the large difference in T90 for two strains when their die-off was studied under dark conditions. Deller *et al*. (2006) studied the influence of depth and UV-B transmittance of freshwater bathing waters on the elimination of bacteria in water and found that: (i) *Enterococcus faecalis* appears to be more resistant to UV-B than *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*); (ii) radiation intensity is significantly correlated with reduction rates on the water surface; and (iii) high turbidity substantially reduces UV-B transmittance in water causing decreased elimination efficiency.

- Temperature. It is a known controller of FIO inactivation; however, FIO dependence on temperature is determined by physio-chemical and ecological factors and is site specific.
- Salinity, with higher salinities increasing die-off rates. Salinity influences can be neglected at saline concentrations less than 15 g L^{-1}, but that these influences become substantial when salinity levels approach concentrations characteristic of marine waters. This explains why the upper limit of the area of influence is considered to be the head-of-tide region. The hostile situation encountered by FIO in seawater can trigger cells to enter a viable-but-non-culturable state.
- Dissolved oxygen concentrations substantially affect FIO in-stream. However, within eutrophic waters, algae may reduce or enhance FIO die-off caused by sunlight, since they both impede light penetration and increase oxygen concentrations.
- pH effect. FIO die-off in surface waters increase when conditions become acidic (pH< 6) or alkaline (pH>8).
- Suspended particulate matter (SPM). In marine environments, the greater the SPM concentration the lower the die-off rate of FIO; however, there is limited information on the FIO-SPM interaction in freshwater systems, with few recent studies indicating that SPM concentrations increase decay rates of FIO in brackish waters but have minimal or inconsistent influence in freshwater environments.

Survival periods in groundwater are 20-30 days for FC (Crites and Tchobanoglous 1998 cited in Richards *et al.*, 2016a) and 90-110 days for *E. coli* (Flint 1987).

3.9 FIO monitoring techniques and technologies (Objectives 1-2)

Current and emerging monitoring technologies enable measurements of FIO using the following methods (see detailed description in Appendix IV):

- Cultivation-based techniques that enumerate FIO grown on selective media, e.g. membrane filtration, Coliscan Easygel system, MI agar, Chromocult coliform agar, membrane lactose glucuronide agar (MLGA)) and Colilert.
- Enzyme-based (e.g. activity of β-d-glucuronidase) or respiration-based techniques measuring FIO or total bacterial metabolic activity, respectively, e.g. MWK 1.0 lab testing kit, Bacti-Wader, Bactiquant Water, BACTcontrol, Microbial Bioanalyser, ColiMinder and Speedy Breedy.
- Molecular-based techniques (DNA or RNA) that may involve targeting genetic biomarkers for FIO (e.g. the 16S rRNA gene in *Bacteroides*, host mitochondrial DNA or virus tail protein genes) or variations in genomic characteristics (e.g. genome lengths), e.g. paper-origami DNA microfluidics, RNA biosensors, QPCR, microarray, antibiotic resistance, ribotyping, pulse-field gel electrophoresis, denaturing gradient gel electrophoresis, rep-PCR, length heterogeneity PCR and terminal restriction fragment length polymorphism.
- Cell counting/sorting using visual inspection (sometimes using fluorescence) and subsequent enumeration of cells, e.g. flow cytometry and FACS, BactoSense and BACMON.

Chemical indicators (nitrogen, ammonia, caffeine, anionic surfactant, fluoride, fluorescence whitening agent, faecal sterol and acid bile markers) and physio-chemical changes (turbidity, conductivity and temperature) that can be used as proxy measurements for FIO.

The technologies can be implemented in the following ways:

- In the lab: samples are required to be taken back to the lab for analysis. This sampling can involve spot sampling or autosamplers and is not further discussed in this report. It is standard practice to transport samples on ice, but transportation of samples and storage causes issues with FIO die-off (see Section 3.10.1). Examples of these technologies include: membrane filtration techniques, Coliscan Easygel system, Colilert, MI Agar, Chromocult Coliform Agar, MGLA, faecal sterol and acid bile markers, chemical indicators including ammonia, flow cytometry and fluorescent activated cell sorting (FACS), paper-origami DNA microfluidics and RNA biosensors, Bactiquant Water, QPCR, microarray, antibiotic resistance, ribotyping, pulse-field gel electrophoresis, denaturing gradient gel electrophoresis, rep-PCR, length heterogeneity PCR and terminal restriction fragment length polymorphism.

- In the field as mobile labs or devices: samples are manually applied to the monitoring device on site and provide results within minutes to hours. Examples of mobile labs include: Aquaflex (Trace2O), compartment bag test (Aquagenx), Colitag (Palitest), Aquacheck365, MWK 1.0 lab testing kit and Microbial Bioanalyser. In the case of probes (e.g. for temperature, conductivity or turbidity), manual extraction of samples is not required, and the probe is applied directly to the water.
- Continuous monitoring: the monitoring device is left *in situ* and measurements are taken continuously and, in some cases, results can be sent to a mobile device or PC via SMS text message or email, respectively. This is especially useful for remote sites. Examples of these technologies include: Bactosense[9], ALERT E. coli Analyser.

Many bacterial species can become dormant as a strategy to survive harsh environmental conditions (Lennon and Jones 2011). Such cells are known as viable but non culturable (VBNC) and may confound FIO measurements, depending on technology. Proxy measurements that do not rely on the presence of bacterial cells are not affected by the presence of VBNC. However, cultivation-based technologies, particularly library-dependent methods (Box 2), are unsuitable for the enumeration and characterisation of VBNC. In the VBNC state, bacteria maintain a low metabolic activity, but do not divide (e.g. Oliver *et al.*, 2005); therefore, technologies relying on the metabolic activities of bacterial cells may pick up VBNC depending on their limit of detection. For example, flow cytometry, which is based on staining and visualisation of cells can distinguish and enumerate viable and VBNC cells from dead cells provided that appropriate staining methods are used (Khan *et al.*, 2010).

DNA-based methods such as qPCR can over-estimate FIO measurements because they allow detection of both live and dead cells. Previous studies have demonstrated that PCR-amplifiable DNA can persist in dead bacterial cells and as free molecules in natural waters (Kreader, 1998; Bae *et al*., 2009). This poses an issue when measuring FIO due to the introduction of false positives, and results may not correlate with levels of recent contamination events, identification of predominant pollution sources and the fate and transport of FIO (Kapoor *et al*., 2015). This could be overcome by the addition of intercalating dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA), which allow only intact cells to be enumerated (Nogva *et al.*, 2003; Nocker *et al.*, 2006). However, such dyes do not distinguish between metabolically active cells, and so enumeration of microbial rRNA precursors (pre-rRNA) has been suggested as an alternative enabling the rapid detection of viable cells, including VBNC (Lee and Bae 2018).

Previous studies have also shown that the qPCR method:

(i) Can overestimate viable cell quantities in the logarithmic phase of growth (Ludwig and Schleifer 2000)

(ii) Is less affected than culture-based methods by collection time, potentially as a result of different persistence and sensitivity to light of molecular materials versus viable culture cells (Wade *et al.*, 2005; USEPA, 2010; Haugland *et al.*, 2005).

(iii) Is less sensitive than culture-based methods to environmental factors such as rain and turbidity (Telech *et al*., 2009; Haugland *et al.*, 2005).

It must also be noted that a study of tracers for STS and human FIO in Scotland showed that (Richards *et al.*, 2017):

- Fluorescence detection of human FIO from STS can be limited to water courses with low level of dilution or to streams with extreme low discharge.
- For streams with high level of dilution, tracking STS discharges using caffeine and artificial sweeteners can be effective but it involves complex, expensive and labour intensive techniques.

Water quality parameters such as turbidity, ammonia, conductivity and temperature can provide indirect evidence of the presence of wastewater effluent. For example: temperature sensing can help identify point sources of relatively cooler or warmer water into a stream; conductivity sensing can identify point sources of wastewater with relatively higher conductivity into a stream; and turbidity can identify sources of wastewater with relatively higher turbidity. It also important to recognise that these parameters influence FIO survival and are not linearly related to FIO concentrations in the water environment (Section 3.8).

That said, the problem with using these parameters as proxies for FIO is that their relationship with FIO remains study-specific (Appendix IV). Nnane *et al*. (2011) studied microbial water quality at the River Ouse catchment in southeast England (U.K.) and found that turbidity could act as a surrogate for presumptive *E. coli* levels, and presumptive intestinal enterococci, under many conditions. Based on their results, Nnane *et al*. (2011)

9 This is not a continuous method but it can transmit results.

suggested using turbidity as a cheaper and simpler surrogate for FIOs, if initial data suggest that turbidity correlates significantly with levels of FIO in the study area. However, SEPA's investigations in Scottish rivers concluded lack of significant correlation between FIO and turbidity. Potentially, the different sensitivity of each FIO technology to environmental factors is responsible for the inconsistent relationship between FIO and water quality parameters such as temperature, conductivity, ammonia and turbidity. In this context, it would be useful to collect data on turbidity as well as ammonia, conductivity and temperature, if possible, to help better understand how environmental factors directly or indirectly influence FIO detection with different FIO technologies and at different environmental conditions.

The technologies were split into two categories: 'best current technologies' and 'most promising emerging technologies' (Table 5a). There is limited published information for emerging technologies as few studies have tested them. Further, the information available on the manufacturer's websites may be biased. Given that we have no personal experience of using emerging technologies, caution must be taken when considering the literature review findings on their applicability (Appendix IV). The technologies were also grouped according to their relevance to the phase of monitoring (see Section 3.11).

3.9.1 Faecal source tracking

Faecal source tracking by microbial or chemical methods is key to targeting effective FIO control management practices for downgraded BWPA and SWPA (Dufour *et al.*, 2012; Rees *et al.*, 2010). It includes several host-specific microbial and chemical methods which have the potential to determine whether diffuse sources of faecal pollution are human or non-human, and distinguish between non-human faecal sources (Gourmelon *et al.*, 2010; Dufour and Bartram, 2012). Microbial markers (their characteristics are detailed in Appendix Table IV.1) of faecal sources include library-dependent and library-independent methods (see Box 2). Chemical markers of faecal sources include (their characteristics are detailed in Appendix Table IV.1): faecal sterol, acid bile markers, ammonia, nitrogen, caffeine, anionic surfactant, fluoride and fluorescence whitening agent. An example of the relationship between microbial source tracking markers and flow is given in Box 3.

Table 5a. List of current and emerging technologies

Current Technologies/ Methods	Emerging Technologies
Membrane filtration techniques	aquaCHECK356 (Brighwater Diagnostics)
Coliscan Easygel® System	Bacti-Wader (Chelsea Technologies Group Ltd)
Colilert	MWK 1.0 Lab Testing Kit (Glacierclean Technologies Inc)
MI agar	Paper-Origami DNA microfluidics
Chromocult agar	RNA biosensors
Membrane lactose glucuronide agar (MGLA)	Flow cytometry (for tracking FIO) and FACS*
AquaFlex (Trace2O)	Microarray
Compartment Bag Test (Aquagenx)	Bactiquant Water (Mycometer)
Colitag (Palintest)	BactoSense
Temperature sensing	BACTcontrol (MicroLAN)
Conductivity sensing	Microbial Bioanalyser (Photonic Biosystems)
Turbidity measurements	BACMON (GRUNDFOS)
Faecol sterol and acid bile marker detection	ALERT – *E. coli* Analyser (Planet Ocean Ltd)
Chemical indicators (e.g. caffeine) detection	ColiMinder CMI-01 (VWR Solutions)
Ammonia measurements	Speedy Breedy
Quantitative PCR (qPCR)	
Antibiotic resistance testing	
Ribotyping	
Pulse Field Gel Electrophoresis (PFGE)	
Denaturing Gradient Gel Electrophoresis (DGGE)	
Repetitive DNA sequences PCR (Rep-PCR)	
Length heterogeneity PCR (LH-PCR)	
Terminal restriction fragment length polymorphism (T-RFLP)	

*FACS: Fluorescent Activated Cell Sorting.

Box 2. Library dependent and library independent microbial source tracking tools.

- Microbial source tracking (MST) tools are broadly split into library-dependent i.e. requires the cultivation of organisms to make a library, followed by characterisation of those organisms; and library-independent i.e. characterisation of organisms in environmental samples using total genomic DNA extracted from those samples.
- Library-independent techniques involve the detection/quantification of specific host-associated gene targets. Current techniques include QPCR, TRFLP and DGGE, though the latter two have fallen out of favour. Emerging techniques include microarrays and next generation sequencing.
- For assays involving PCR, human-specific gene targets are more well developed than those targeting animals because contamination with human faeces is regarded as a critical health issue. Gene targets need to be present in high concentrations to mitigate against dilution effects. They also need to have a long half-life in the environment.

Source: Pagaling and Avery, 2017

Box 3. Faecal source tracking at different flow regimes

A study of FIO in 16 small stream sites in Wisconsin found higher maximum faecal coliform (FC) concentrations in stream samples collected during baseflow (58 X 10^3 cfu/100ml) than during stormflow (6.6 X 10^3 cfu/100ml). Median FC concentrations indicated no consistent response in relation to flow but there was a positive relationship with increasing urban land use. Similar patterns were observed for *E. coli* and coliphages. Serotyping RNA coliphages enabled FIO source tracking. Detection frequency (DF) of serogroups indicating human sources suggested that human faecal sources were positively correlated with urban land use. Human DF was higher during baseflow than during stormflow and higher in the autumn than in the spring. When flows were combined with seasonality, DF of human faecal sources was higher during baseflow than during stormflow for the same season.

Source: Thomas *et al*., 2017

Until now, no single source tracking microbial or chemical method appears as the "best" method to identify the origin of faecal pollution in water (Gourmelon *et al.*, 2010; Dufour *et al.*, 2012; Harwood *et al.*, 2016; Pagaling and Avery 2017; Garcia-Aljaro *et al.*, 2019). A 'toolbox' approach combining several microbial and chemical analytical methods could potentially improve certainty in faecal source tracking (Pagaling and Avery 2017; Gourmelon *et al.*, 2010 and literature cited therein; Garcia-ALjaro *et al.*, 2019). Box 4 outlines the basic components of the toolbox approach. It must also be noted that a toolbox of assessment techniques combining desktop surveys, FIO monitoring, modelling and multiple host-specific markers is the best approach to understanding and predicting pathogen exposure (Dufour *et al.*, 2012; KInzelman and Ahmed 2015; USEPA 2010; Thomas *et al.*, 2007). This is further discussed in Section 3.11 (monitoring strategy).

Box 4. Basic components of a toolbox approach to faecal source tracking

- No single faecal source tracking assay is perfect. Therefore, a toolbox approach is required where several markers are detected/quantified in the environment. Two approaches were identified:

  1. A targeted approach based on land use data, which may involve applying a 'toolbox' of assays selected according to land use data e.g. urban-impacted tested for human and domestic animals; upland waters tested for sheep and deer; and waters near wetlands tested for wild birds.

  2. Initial screening followed by a more in-depth analysis, which may include a general presence/absence assay for an initial screening of FIO and, based on results, selection of samples for quantitative host-specific assays.
- An expert opinion survey showed that there was no clear consensus of the 'best' MST tool, and the use of multiple markers (i.e. toolbox) was highlighted several times. It was noted that the importance of sampling regimes and measurement of catchment variables is often neglected, and that modelling aspects and risk-assessment approaches are also important. Another consideration was the age of the faecal pollution.

Source: Pagaling and Avery, 2017.

3.10 FIO monitoring frequency considerations (Objective 3)

Managing and developing meaningful monitoring programs for optimal water quality require sound scientific data on the variability of faecal contaminants (e.g. microorganisms and nutrients), their concentrations, and their sources (Whitman and Nevers 2004; Meays *et al.*, 2006). The release of FIO from sediments must also be estimated in order to distinguish historical faecal contamination (legacy FIO) from recent contamination; otherwise, recommendations to change management practices may be misguided (Pachepsky *et al.*, 2017). Monitoring must also address the impacts of stream characteristics on FIO spatial and temporal variability, including but not limited to factors such as flow, river length, exposure to sunlight, temperature, and faecal reservoirs, as well as ecological processes, including the effect of grazing on the distance *E. coli* can travel in different catchments (Meays *et al.*, 2006; Harmel *et al.*, 2016).

The main FIO monitoring concerns are related to capturing the effect of:

- Factors influencing spatial variability of FIO pollution within catchments.
- Factors influencing temporal variability of FIO pollution within catchments.

Factors influencing spatial variability of FIO pollution within catchments.

- FIO are expected to be higher near the bottom than at the surface during both baseflow and stormflow (USEPA 2010). Therefore, stream water samples must consistently be collected near bottom to capture maxima FIO concentrations or near the surface to capture the effect of surface water plumes.
- Except in the immediate vicinity of point sources, FIO gradients in the downstream direction will be determined not only by dilution but also by the factors influencing survival and enrichment from transit FIO reservoirs such as streambed and streambanks (USEPA 2010; see also Section 3.1). Therefore, investigative sampling must explore the potential of FIO in streambed and streambanks to confound stream water monitoring results.

Factors influencing temporal variability of FIO pollution within catchments

Temporal variability in FIO concentrations—at time scales ranging from minutes to months—has been observed in time series analysis of FIO (US EPA 2010). In this context:

- Variations with time scales in the order of minutes are important because such considerations influence the number of samples needed to accurately characterize microbial water quality and the confidence with which to ascribe results of sampling events.
- Variations with times scales in the order of tens of minutes are important because they have the same time scale as that of typical recreational use episodes.
- Variations with time scales in the order of a day are important because their knowledge allows comparison between samples taken at different times of the day or between samples taken on successive days.

Accounting for diurnal variations in FIO concentrations requires (Whitman and Nevers 2004):

- Collecting samples at a standard time of day at which maximum exposure to the general public is anticipated.
- Using early morning samples for developing conservative estimates (i.e. accounting for the effect of solar radiation, which is a certain effect) of water quality.
- Using adaptive sampling (collecting supplemental samples based on the results of earlier sampling events).

3.10.1 FIO sample storage – Results from unpublished experiments

Storage time is a particularly important issue for remote sites where it may take longer than 24h to transport samples back to the lab.

Regulatory requirements for FIO sample storage and laboratory analyses. Specific regulations specify the procedures required for FIO sampling at BWPA and SWPA[10] and for laboratory analyses. For example, The Bathing Waters (Sampling and Analysis) (Scotland) Directions 2008 (hereafter reported as BWPA Directions) provide specifications about sampling and analysis of the bacteriological samples collected in a BWPA. A key requirement of the BWPA Directions is that the interval between sampling and laboratory analysis should not exceed four hours. Where this is not possible, SEPA must store the sample(s) in a refrigerator and keep this interval as short as possible, and no longer than 24 hours. Further, if it is not possible to apply the BWPA Directions, SEPA may use such rules that it considers substantially equivalent to the BWPA Directions, provided they have notified the Scottish Ministers giving details of such rules and methods and their equivalence. However, there is no legal obligation for the BWPA Directions to be applied to investigative bacteriological monitoring (e.g. in-stream monitoring as part of a BW profile). Robust

10 The majority of SWPA are currently monitored by Food Standard Scotland (FSS); in this respect, SWPA monitoring guidance is beyond the scope of this report.

and practical alternatives to the storage time specified in the BWPA Directions are needed to investigate faecal pollution sources in remote, upland waterbodies in BWPA catchments.

Alternative options for FIO storage time based on unpublished experiments. Water samples collected from 6 different sites in Scotland (L. Avery, unpublished data generated through funding from the Rural and Environment Science and Analytical Services-RESAS Strategic Research Programme) suggests that *E. coli* and TC concentrations may be fairly stable during the first 72h after sampling but that samples should not be stored for longer periods before laboratory analysis, which was done using Colilert in these experiments. Appendix VI presents summary graphs from these experiments. Storage time can affect quantification of FIO depending on the technology used (Appendix Table IV.2). Technologies that rely on genetic material are affected by storage time as DNA and RNA will degrade over time; RNA loss is more rapid than DNA loss. DNA loss is further complicated by species e.g. *Bacteroides* do not survive for long periods in water; as the cell degrades, DNA is released, and free DNA is broken down rapidly. Technologies that rely on whole cell counting will be affected by storage time in the same way that cultivation-based methods are as these technologies work on undamaged cells. Furthermore, technologies that measure stanols and sterols are affected by storage time as these chemicals change over time. However, we found no published literature on FIO monitoring mentioning storage time longer than 24h for cultivated-based or DNA-based methods. For example, Telech *et al*. (2009) report that they performed membrane filtration and qPCR in the lab 6h after sampling while Wyer *et al*. (2010) performed cultivation-based methods in the lab within 24h of sampling.

3.10.2 Sampling frequency by technology

Frequency of sampling for a given current or emerging FIO technology depends on the purpose of sampling and knowledge of in-stream FIO variability at different scales at a given site and time (e.g. diurnal, event-scale, seasonal, and stream-reach scale). However, sampling frequency per FIO technology remains briefly addressed in the literature. We summarise available evidence-base below.

Cultivation-based methods

Cultivation-based methods applied in the lab or in-field are sensitive to diurnal variability showing higher FIO in the morning and evening than mid-day and to environmental factors such as rain, and turbidity (USEPA 2010); see section 3.8. Previous studies for FIO detection with these technologies have collected samples as follows:

- Accounting for diurnal variability requires sampling three times a day, e.g. 8 am, 11 am, and 3 pm (Telech *et al.* 2009).
- Detecting catchment-based FIO hotspots requires twice weekly sampling or hourly if connectivity between source and receiving protected areas is high (Kay *et al.*, 2010; Wyer *et al.*, 2010).
- Accounting for event-scale and variability at the stream-reach scale requires replicate sampling to account for any effect of small-scale variability (e.g. hourly sampling for 2-3 consecutive days or weekly sampling for a month or longer during initial screening); or composite sampling with autosamplers to capture the full range of diurnal and event-scale variability (Stocker *et al.*, 2018; Jordan and Cassidy, 2011; US EPA, 2010).
- Accounting for FIO variability due to rainfall and subsequent runoff requires concurrent sampling of in-stream FIO concentrations and discharge and a sampling design that encompasses a range of flows using spot sampling or autosamplers (Kay *et al.*, 2008a; b; Kay *et al.*, 2010). This requirement has been extensively discussed in an earlier CREW report (Akoumianaki *et al.*, 2016b) providing monitoring guidance to SEPA on collecting stream samples with autosamplers.
- Understanding background FIO and separating rainfall-independent (continuous) from rainfall-dependent and emergency FIO discharges requires sampling during both wet and dry weather. Hereafter, this monitoring design is reported as hybrid design. This design is ideal for assessing the differences between wet versus dry weather FIO concentrations in-stream through collecting dry weather monitoring and high frequency monitoring data during events (hence hybrid monitoring).

DNA-based methods

- DNA-based methods are less sensitive than cultivation-based methods to diurnal variability and environmental factors (see section 3.9). As such, many studies determining faecal contamination of surface waters using molecular markers use a weekly sampling frequency (e.g. Hamza *et al.* 2011, Bofill-Mas *et al.* 2010). MST markers are also a good indicator of faecal pollution as they can apportion the main sources. Source apportionment was not a requirement for this objective, however, one-off sampling for MST can elucidate predominant FIO sources, but should only be performed as confirmatory or hypothesis-driven sampling following surveys for the detection of FIO hotspots and knowledge of the timing of FIO discharges at a given site and season (McDonald *et al.*, 2006; Telech *et al.*, 2009).

Bacti-meter (measures tryptophan)

No literature was found regarding the sampling frequency required to find main FIO sources in surface water using tryptophan as a marker. All literature found using tryptophan as an indication of faecal contamination was for groundwater and drinking water (e.g. Sorensen *et al.* 2015, Nowicki *et al.* 2019).

Flow Cytometry

No literature was found regarding the sampling frequency required to find main FIO sources in surface water using flow cytometry, presumably because the use of flow cytometry for FIO monitoring is relatively new.

Proxy measurements - Tracers

- Sampling of proxy measurements, biomarkers and chemical indicators for the detection of FIO hotspots can be low frequency (e.g. four times a year) to high frequency (e.g. twice weekly). For example, Nnane *et al.* (2011) used twice weekly year-round sampling for conductivity, ammonia and turbidity in order to detect catchment-based hotspots. Richards *et al.* (2017) showed that a combination of multiple tracers and indicators are the most indicative way of tracking STS discharges and that factors such as rainfall, stream discharge and volume needed for analysis are important to consider before choosing a tracer.

Number of samples

Number of samples or sample size may refer to replicates per sampling station and to number of samples seasonally or annually. Sample size depends on background variability and certainty required to predict risk to public health. Extensive guidance on sample size and how to estimate it has been provided to SEPA in two earlier CREW reports (Akoumianaki *et al.*, 2016a; b).

3.11 FIO monitoring strategy

The EU guidance on catchment surveys in BW and SW catchments (see Appendices I.3 and I.4, respectively) provide a practical blueprint for undertaking microbiological surveys. The key idea in EU guidance is to start with initial surveys aiming to identify the main FIO sources in the area of influence and thereafter, carry on with a closer examination of the main FIO sources mainly involving source-specific monitoring to capture temporal variation or use of historical data (where available) to understand FIO dynamics. However, the EU guidance fails to provide explicit guidelines on the design of field surveys and investigative monitoring programmes to help detect FIO sources in order to inform their control as part of the RBMP process. Further, sampling for faecal source tracking in bathing waters and in shellfish flesh is not precluded but there are no guidelines for siting monitoring stations to detect locations of predominant FIO sources.

Here, we summarise the findings of a review of monitoring strategies to detect FIO sources, which were developed to inform management of bathing and shellfish waters. It is important to note that there is a broad consensus in the international literature that the monitoring strategy to detect and assess FIO sources within catchments involves three phases.

- The first phase involves catchment surveys, ideally but not necessarily accompanied by initial FIO monitoring to identify FIO hotspots (i.e. sites posing the greatest risk of faecal pollution to bathing and shellfish waters) within the area of influence.
- The second phase includes monitoring (and potentially modelling) within the area of influence to further specify and understand temporal variability in discharges and impact from FIO hotspots. The phase is increasingly applied as confirmatory or hypothesis-driven monitoring to identify the predominant FIO sources (i.e. human, livestock, wildlife or pets) within the area of influence using microbial and chemical source tracking tools.

This phased approach to monitoring to detect predominant catchment-based FIO sources is in line with EU guidance (see Appendices I. 4and 5). The following sections summarise the key points of the monitoring strategy described in (unless otherwise stated): EPA Victoria 2007; Mattl *et al.*, 2009; Nnane *et al.*, 2011; Dufour *et al.*, 2012; Kinzelmann and Ahmed 2015; US EPA 2010; USEPA-NSCEP 2013; Disney *et al.*, 2014; Lindberg 2010; Miller and Dorn 2016; Neil *et al.*, 2018; Gourmelon *et al.*, 2010; Reischer *et al.*, 2011; Dufour *et al.*, 2012; Kinzelmann and Ahmed 2015; Miller and Dorn 2016; Sims and Kaczor 2017; Richards *et al.*, 2017.

It must also be noted that at the end of the literature-based guidelines for each phase of the FIO monitoring strategy, we provide our recommendations to SEPA.

3.11.1 Phase 1: Catchment surveys to identify area of influence and main FIO sources

Phase 1 involves catchment surveys to determine the area of influence in a particular area and to detect the main FIO sources therein. In this respect, catchment surveys are key to developing a monitoring programme that is fit-for-purpose.

(i) Approaches to catchment surveys

Catchment surveys may include the following approaches:

1. **Field screening or field surveys.** These involve physical inspections (with or without initial

monitoring) to characterise FIO load in discharges and their frequency, which can help to characterise the degree of FIO problems at a waterbody- or river-catchment scale or for a specific type of source.

- o Major problems may refer to relatively large FIO inputs to watercourses or high load in point-source discharges, occurring frequently.
- o Moderate problems may refer to relatively medium FIO inputs to watercourses or moderate load in point-source discharges occurring intermittently. Further, moderate problems may arise from FIO discharges flowing through flowing through a riparian buffer or a soakaway.
- o Minor problems may refer to relatively small FIO inputs or load in discharges infrequently occurring, not transported off-site, and easy to control.

2. **Desktop studies.** These involve use of published or historical data, mapping, GIS analyses or GIS-based risk mapping (Box 5).
3. **Modelling.** This refers to any tool that predicts FIO hotspots based on livestock numbers and land use and FIO dynamics and decay during transport from catchment-based sources to the coast (Box 6).
4. **A combination of the above, hereafter called a "toolbox approach to catchment surveys".** This approach is ideal when each of the above approaches can contribute supplementary lines of evidence towards a better understanding of the processes and activities contributing to FIO pollution in a catchment and the coastal zone.

Figure 4 summarises the advantages and disadvantages of these approaches.

Box 5. Desktop-studies

These refer to use of published evidence, maps and GIS data and analyses. They can be stand-alone when the evidence base and digitised data are up-to-date and when baseline-catchment walks for visual observations have already taken place. Thus, in data-rich circumstances, desktop studies can be a cost-effective, stand-alone approach to catchment surveys and to siting FIO monitoring sites. When desk-based catchment assessments are designed to provide output that can be subjected to peer review (e.g. maps, database of georeferenced data), they can be used by multiple agencies or stakeholders to justify catchment monitoring and investments. Field observations are also essential to enable verification or updating of digital data.

The following types of data can inform desktop studies:

- Published data on specific FIO inputs at specific location within the river network or to the coast, or FIO load in the discharges of specific sources (e.g. untreated sewage, wildlife).
- Expert judgement, when experts have site-specific knowledge of the processes influencing FIO inputs to watercourses and FIO delivery from sources to sea. Equally, expert understanding of the knowledge gaps and uncertainties pertaining to FIO dynamics in catchments is key to developing assumptions for scoping available evidence.
- Maps of georeferenced locations of actual and potential point sources.
- Maps of the river network and, if possible, of the road and sewerage network.
- GIS maps of land use/land cover and designated areas.
- GIS-based risk mapping, which can help to inform siting of all locations where FIO may enter the hydrological network leading to the BWPA or SWPA. This may include soil maps indicating risk of leaching and runoff as well as flood risk maps and diffuse pollution risk maps.
- GIS-based estimates of the distance of septic tanks or other sewage sources (e.g. livestock intensive installations).

Box 6. Modelling as an alternative to monitoring

- Process-based modelling requires a significant amount of data for model parameterisation and validation (Porter et al., 2017). However, there is scarcity of data on FIO concentrations in-stream and fluxes compared to nutrient and sediment flux (Muirhead, 2015, Oliver et al., 2016).
- Existing fate and transport predictive models, such as SWAT (Gassman et al., 2007) and HSPF (Bicknell et al., 1997 cited in Pachepsky et al., 2006) account for E. coli survival by estimating survival rates.
- There are knowledge gaps regarding the complex behaviour of FIO persistence in different matrices such as faecal deposits (Soupir et al., 2008, Martinez et al., 2013, Oliver and Page, 2016), soil (Muirhead and Littlejohn, 2009, Park et al., 2016) and streambed sediment (Pachepsky and Shelton, 2011, Shelton et al., 2014, Pandey and Soupir, 2014, Pandey et al., 2018). These gaps make it difficult for all processes to be considered in complex process-based models (Beven, 2006, Cho et al., 2016a; b).
- Risk assessment tools that can identify 'hotspots' of FIO pollution in catchment systems are welcomed by regulatory agencies as a mechanism to help understand origins of pollution and to spatially target catchment management and interventions for improvements in microbiological water quality (Dymond et al., 2016 cited in Porter et al., 2017).
- The Sensitive Catchment Integrated Mapping Analysis Platform (SCIMAP) has demonstrated significant potential as a framework to inform on catchment-scale risks for diffuse nutrient and sediment pollution (Reaney et al., 2011) and more recently for FIO (Porter et al., 2017; Oliver 2018).
- The SCIMAP approach is underpinned by the source-mobilisation-delivery-impact (SMDI) continuum and critical source area (CSA) concepts (Haygarth et al., 2005), which describe how a source of pollution can only convert to a pollution risk if there are no interruptions to the SMDI continuum.
- SCIMAP-FIO is a decision support tool which predicts where the E. coli is coming from and where it is likely to go in the river network (Oliver et al., 2018; Reaney et al., 2017). The output of the decision-making tool SCIMAP-FIO for livestock[11] allows users to identify the source areas within a landscape that are most likely to be contributing FIO as diffuse pollution and may be the sources of the pollution increasing in-stream FIO concentrations. The approach is based on the combination of two existing modelling approaches; SCIMAP, which models how runoff will flow across landscapes and reveals the connectivity of land and waterways; and the Visualising Pathogen & Environmental Risk (ViPER) model, which predicts the accumulation of E. coli contamination on landscapes, such as pasture land (Oliver 2018).

11 Two members of the project team attended SCIMAP-FIO user group meetings: Ioanna Akoumianaki attended a user group meeting held in Durham University in February 2018. Eulyn Pagaling attended a user group meeting held in Birmingham in September 2018.

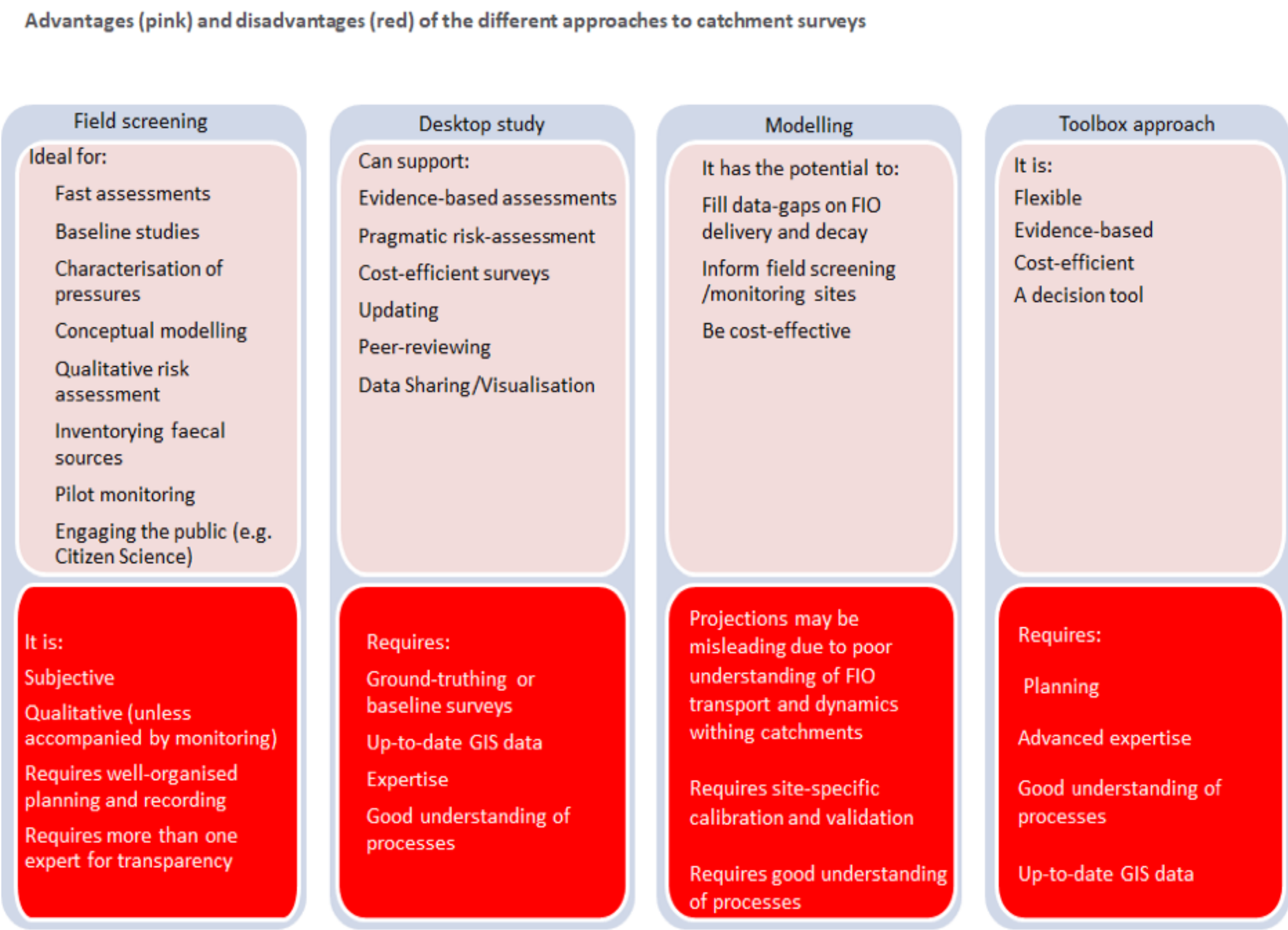

Figure 4. Advantages and disadvantages of approaches to catchment surveys.

(ii) Identifying the area of influence and the main FIO sources during physical inspections

Identifying how far upstream from bathing or shellfish waters and how far inland from rivers, streams and coastline to undertake the catchment surveys is key to delineating reliably the area of influence. Point sources such as WwTW are relatively easy to spot and test if they indeed represent FIO hotspots, i.e. sources of FIO pollution to BWPA and SWPA. Catchment surveys must also enable the identification of areas acting as diffuse FIO pollution hotspots (aka as "areas of concern" in USEPA-NSCEP 2013). Such areas may include agricultural land, areas with clusters of domestic septic tanks and areas accumulating wildlife or pet faeces. Identifying sites within the river network acting as hotspots may require elaborate monitoring, such as that prescribed for Phase 2 (Section 3.7.2).

Literature-based advice to staff undertaking or organising catchment surveys is detailed below.

1. Start at the shoreline and progressively move up to the catchment gradually, prioritising human sources and "areas of concern" in each catchment.
2. Apply a **tiered approach** to identify how far upstream to undertake surveys, as follows.

Tier 1

Start with areas where FIO concentrations have been documented (i.e. where published or historical FIO data are available) and any other FIO sources explored through investigative monitoring or research studies. Data are usually available at and in the vicinity of BWPA and SWPA through routine monitoring and regulatory inspections and in the vicinity of point-source discharges.

Tier 2

Account for FIO sources known to have a direct impact on coastal water quality, i.e. WwTW discharges directly to BWPA and SWPA or within the waterbody catchment in the immediate vicinity of BWPA and SWPA (i.e. the coastal waterbody catchment). FIO sampling is not necessary because FIO pollution risk can be assessed based on knowledge or visual inspections of types of point and diffuse sources which can help establish the degree of problem (i.e. major, moderate or minor).

Tier 3

Prioritise inspections on FIO sources and areas of concern located in the immediate vicinity of watercourses (between 10 and 50m from rivers, streams, or the coastline, depending on local policy[12]) and on areas where there is a higher risk of runoff, soil leaching or flooding. Sources away from watercourses should be assessed last and only if FIO pollution sources have not been detected nearer to watercourses. Locations of septic tanks in relation to their proximity to the watercourses and the risk of runoff, leaching and flooding as well as diffuse pollution risk from agriculture and wildlife can be identified though desktop, GIS-based analyses. Locations of point sources are usually known to environmental agencies. However, locations of domestic septic tanks or areas of concern are not always well documented. The advice for these cases is to bracket (i.e. monitor upstream and downstream) potential FIO sources. For example, if monitoring downstream a suspect area (e.g. standing water or seepage near rivers and streams) shows FIO levels that are higher compared to upstream monitoring results, this provides sufficient evidence for further inspection and monitoring to narrow down the FIO source area, and thus detect the location and type of the actual FIO hotspot to inform further remedial action. Monitoring sites should be strategically placed to capture the impact of potential direct and indirect discharges to the rivers and streams and areas of concern. Strategic sites for monitoring may include sites upstream and downstream of:

- Point sources such as WwTW, CSO and stormflow drains and septic tanks.
- Pasture land.
- Arable land and land recently spread with manure, slurry or sewage sludge.
- Areas known for hosting wildlife populations.
- High density residential areas, which may pose a risk from domestic animals.
- Sites of stream confluences with the main stem of a River or stream.

Tier 4

Investigate the upper limit of FIO pollution in the river network draining to the BWPA or SWPA to identify the area of influence. As a rule of thumb, initial inspections of FIO pollution sources can extend up to the head-of-tide-region, if that is known. If available budget permits, and if the upper limit of the tide is unknown, physical inspections (e.g. through windscreen inspections) and initial monitoring sites can be adjusted through monitoring until a "clean" sample indicates that there is no FIO impact from upstream areas.

3. Focus initial sampling on sites and times allowing best and most efficient characterisation, such as sites that:

- Clearly link to faecal pollution, e.g. CSOs are intermittent sources therefore their effect must be monitored during stormflows; septic tanks' soakaways or misconnections are continuous sources but monitoring sites must be immediately downstream of their (potentially) suspected site of discharge.
- Are free of natural sources of faecal pollution, e.g. from wildlife, unless monitoring targets specifically wildlife sources.
- Display small variability during baseflow, in order to reduce bias to assessments especially if this variability is related to the influence from multiple sources at different times of the day or the year. This is explicitly addressed in Section 3.11.2.: Phase 2.

4. Collect samples (during initial sampling) for FIO at morning hours because FIO measured with culture methods demonstrate a predictable pattern of higher density in the morning and minimum density during daylight hours due to solar radiation. Therefore, morning samples can yield conservative results relating to human health effects when using culture methods. Phase 1 monitoring can take place within a limited period of time to indicate which potential FIO hotspots are rainfall-dependent and is conducted during dry and wet weather (hybrid design).

(iii) FIO monitoring technologies for Phase 1

Based on the review of FIO monitoring technologies, we identified initial screening techniques that are fit for in-field exploratory techniques such as the use of proxy measures, or detection of bacterial metabolism using qualitative measures including the following monitoring techniques (Table 5b); see also Section 3.2 and Appendix IV. These measurements can then be confirmed and quantified either in the lab or in the field using an initial FIO assessment by cultivation-based methods (Table 5c).

12 For example, in Scotland the Handbook of Septic Tanks specifies that soakaways should be located at a distance greater than 10 m from watercourses.

Table 5b. Initial FIO screening techniques (Phase 1)	Table 5c. Initial FIO assessment methods (Phase 1)
Temperature	Membrane filtration techniques
Conductivity	Coliscan Easygel® system
Turbidity	Colilert
Bacti-Wader	MI Agar
MWK 1.0 Lab Testing Kit	Chromocult Coliform Agar
	Membrane Lactose Glucuronide Agar (MGLA)
	AquaFlex
	Compartment Bag Test
	aquaCHECK365 / Aquatest
	Colitag
	aquaCHECK365
	Membrane filtration techniques
	Coliscan Easygel® system

3.11.2 Phase 2: Monitoring variability of FIO within catchments

Once catchment surveys have helped to detect the area of influence and the locations where the main FIO sources are located therein, sampling and analyses can take place to account for spatial and temporal variability of in-stream FIO concentrations within the area of influence.

(i) Identifying temporal variability through monitoring

This monitoring (see also Section 3.1.10) can include the designs described below.

- Hourly time-series data can help to assess the effect of diurnal variation in FIO discharges to bathing and shellfish waters. Hourly data can be integrated to form daily composite samples. This sampling is fit for identifying the importance of STS and small WwTW providing secondary treatment in rural areas.
- Weekly time-series in combination with rainfall or flow data and additional evidence on point source discharges and agricultural activity. This monitoring can help to assess variability of in-stream FIO against rainfall-independent, emergency and rainfall-dependent discharges within a short period, e.g. two to three years[13]. It is useful to recognise that we suggest at least two years considering that, in the absence of any historical data in a catchment, two years can be expected to represent variability better than one year alone. This type of sampling is fit for sampling at sites downstream of residential areas, at the confluence of streams draining small waterbody catchments with the main river stem and at BWPA and SWPA.
- Monthly timeseries in combination with rainfall or flow data and additional evidence on point source discharges and agricultural activity. This monitoring can be as useful as weekly monitoring but requires long-term monitoring data to reveal any potential FIO trends with flow and land use.
- Seasonal monitoring. This is fit for seasonality related to tourism, livestock and arable farming.
- Event-based monitoring. Assessing event-scale variability may be challenging in terms of planning prior to monitoring and practical considerations such as sampling, field analyses and transporting samples during stormy weather. However, event-based data may be re-drawn from weekly timeseries monitoring. This monitoring is fit for understanding the impact from rainfall-dependent and emergency FIO discharges (see Section 3.1.5).
- Hybrid monitoring. This monitoring can be applied in relation to CSO, stream-river confluences to assess land use effects, STS clusters and at BWPA and SWPA. This design may require more than two years of monitoring to enable understanding of FIO variability as part of Phase 2 monitoring. This monitoring design can be used during Phase 3 (confirmatory) monitoring to assess dominant FIO sources (see Section 3.3.3).

The literature-based guidance for hourly, weekly, seasonal and event-scale monitoring is to bracket (i.e. monitor upstream and downstream) the locations of the main FIO sources, which were roughly identified in Phase 1. For example, if monitoring downstream a suspect area (e.g. standing water or seepage near rivers and streams) shows FIO levels that are higher compared to upstream monitoring results, this provides sufficient evidence for temporal monitoring to better understand how FIO from that source vary temporally.

13 With regard to accounting for the effect of heavy rainfall events, EU guidance on Bathing Water Profiles suggests: "A selection of samplings on 3-4 years (generally not on a longer period in order to avoid interpretation bias due to heavy changes in infrastructures) will be made by gathering those which are just following storms (day 1 to day 0) and comparing them with results obtained during dry periods" (EEA 2009).

(ii) FIO monitoring technologies for Phase 2

The monitoring techniques (see Appendix IV) that can be used during Phase 2 include those that have been categorised as 'routine monitoring' techniques. Current techniques include the cultivation-based techniques that are fit for Phase 1 and some other proxy measurements such as: faecal sterol and bile markers; chemical indicators (nitrogen, caffeine, anionic surfactant, fluoride, fluorescence whitening agent) and ammonia. However, it must be noted that only human waste can be detected using some of these markers (see Appendix IV). In addition, molecular techniques could be employed during Phase 2, including QPCR and microarray, that adopt the 'toolbox' approach to detecting FIO. The drawbacks of these molecular methods are that they are labour intensive, require expertise and, depending on the primers used, are not 100% specific for the gene target. Flow cytometry[14] is increasingly used to measure total viable counts (TVC) (Scottish Water, pers. comm., February 2019) and has the potential to be used to measure FIO, but to our knowledge, this is not currently used for that purpose.

Amongst the emerging technologies, devices using biosensors are the most promising. These devices mainly refer to paper origami microfluidics and RNA biosensors, which are not available on the market and researchers would have to make them themselves. However, there are several new devices available on the market that are fully automated and so can be used for continuous *in situ* measurements of FIO. In some cases, the devices can be operated remotely, and the results can be sent via SMS text or email. These devices include: BACTcontrol, Microbial Bioanalyser, ALERT, ColiMinder, Speedy Breedy (see also Appendix IV).

3.11.3 Phase 3: Confirmatory testing of predominant FIO sources (microbial source tracking)

(i) Test methods

Phase 3 involves monitoring in the area of influence to elucidate predominant types (i.e. human vs animals) of diffuse FIO sources using microbial and chemical source tracking tools. This monitoring is being increasingly applied as a final confirmatory testing of main FIO types of sources, when the RBMP process requires understanding of diffuse sources of faecal pollution. Here, test methods on source tracking procedures such as faecal sterols analyses, genetic marker testing (e.g. human-specific *Bacteroides*), or genomic DNA profiling using library-dependent MST tools based on those initial results. Once Phase 1 and 2 data have been analysed, decision trees are an effective tool for further planning as well as for selecting the appropriate test method in Phase 3 monitoring (Figure 5).

(ii) FIO monitoring technologies for Phase 3

FIO monitoring technologies suitable for Phase 3 include monitoring of proxy chemical markers and monitoring of genetic markers or whole genomes. All these technologies require transport of samples back to the lab, and so will be affected by storage time. They require expertise and all the molecular methods are labour-intensive. They include: Faecal sterols and acid bile markers, Flow cytometry, QPCR, Microarray, DGGE, TRFLP, Antibiotic Resistance, Ribotyping, PFGE, Rep-PCR, LH-PCR. Technologies for Phase 3 are detailed in Appendix IV.

3.11.4 Trial results

A demonstration of what the toolbox approach to catchment surveys (Phase 1 of the monitoring strategy) can include in Scotland and how it can help to design Phase 2 and Phase 3 of a catchment FIO monitoring strategy is presented in Appendix V. Desktop trials showed that identifying the potential area of influence and siting potential FIO hotspots therein using national published data, maps and GIS analyses accounting for FIO pollution risk from livestock, STS and WwTW is a feasible initial screening alternative to field surveys during Phase 1 of the monitoring strategy.

14 Flow cytometry alone is not an emerging technology but Flow cytometry to measure FIO is an emerging technology.

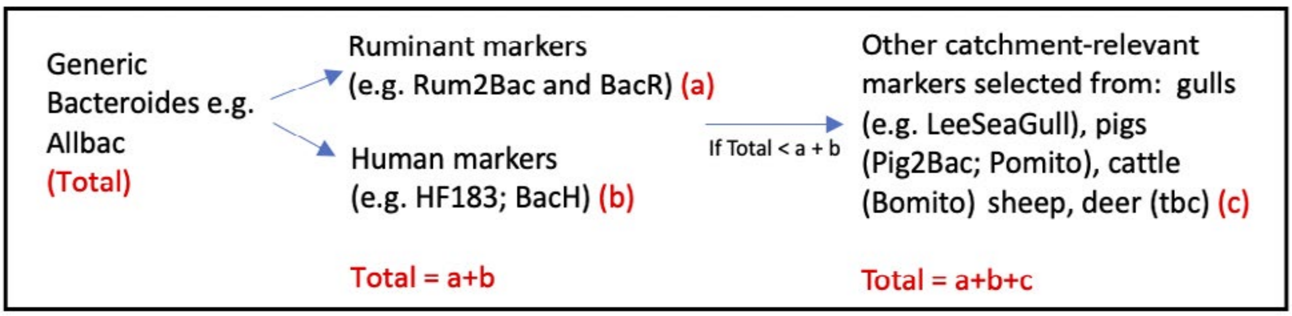

Figure 5. Example of decision tree informing the selection of appropriate test method in Phase 3 of the monitoring strategy. Source: Pagaling and Avery, 2017.

3.12 Visualising diurnal and seasonal variations of in-stream FIO concentrations (Objective 5)

Based on the findings of the literature review, we produced a conceptual graph showing the predominant FIO sources during baseflow and stormflow as well as FIO sources displaying a daily variability (Figure 6). This conceptual graph aims to guide a sampling addressing the evidence on timing of discharges (see Section 3.4 and Figure 2) and in-stream FIO variability (see Section 3.7).

It must be noted that this graph can be used as a broad scenario building tool that could be informed and enhanced by monitoring data rather than as a *de facto* description of the FIO discharges to the river network. This is because FIO discharges and in-stream FIO concentration depend on many site-specific factors, as extensively reported in Section 3.1. It is also useful to keep in mind that catchment features influencing the relationship between FIO concentrations and flow such as size, slope, shape, type of soils vary across Scottish river catchments, therefore any generalisation, such as that presented in Figure 6, should be applied with caution.

4.0 Recommendations

4.1 CREW project team's recommendations for Phase 1 of the monitoring strategy

- **Approach to catchment surveys.** We recommend applying the toolbox approach to catchment surveys because it integrates the advantages of field screening, desktop studies and modelling while being flexible, evidence-based and potentially cost-effective. Its feasibility is tested in the trials presented in Section 4.
- **Monitoring during catchment surveys.** We recommend: starting inspections at the shoreline; inspections and sampling through "bracketing" of monitoring sites; prioritising sampling of human sources or stream-river confluence sites draining areas with human FIO sources; and selecting sites that clearly link to faecal pollution, are wildlife-free, and display small variability during baseflow.
- **Current technologies:** We recommend the use of turbidity measurements and the Colitag mobile lab by Palintest. Turbidity measurements were found to have good correlation with FIO and was subsequently recommended as a surrogate for FIO (Nnane *et al.*, 2011). In addition, this technology is relatively cheap to acquire with no set-up costs, is easy to use and could be used by a non-expert (Appendix IV). Colitag is a mobile lab that consists of sampling vessels, an incubator, MPN plates and UV lamp, which allows growth (on selective media) and enumeration of FIO on site. It is also USEPA's approved method. The initial cost of the lab is estimated to exceed £2K, but it is generally considered as a relatively cheap technology, and easy to use by non-expert staff. Turbidity measurements and the Colitag mobile lab by Palintest can be used in remote sites. We also recommend that these technologies be used in combination, i.e. use the turbidity probe to find out where in the catchment to sample, and then use Colitag to get FIO evidence.

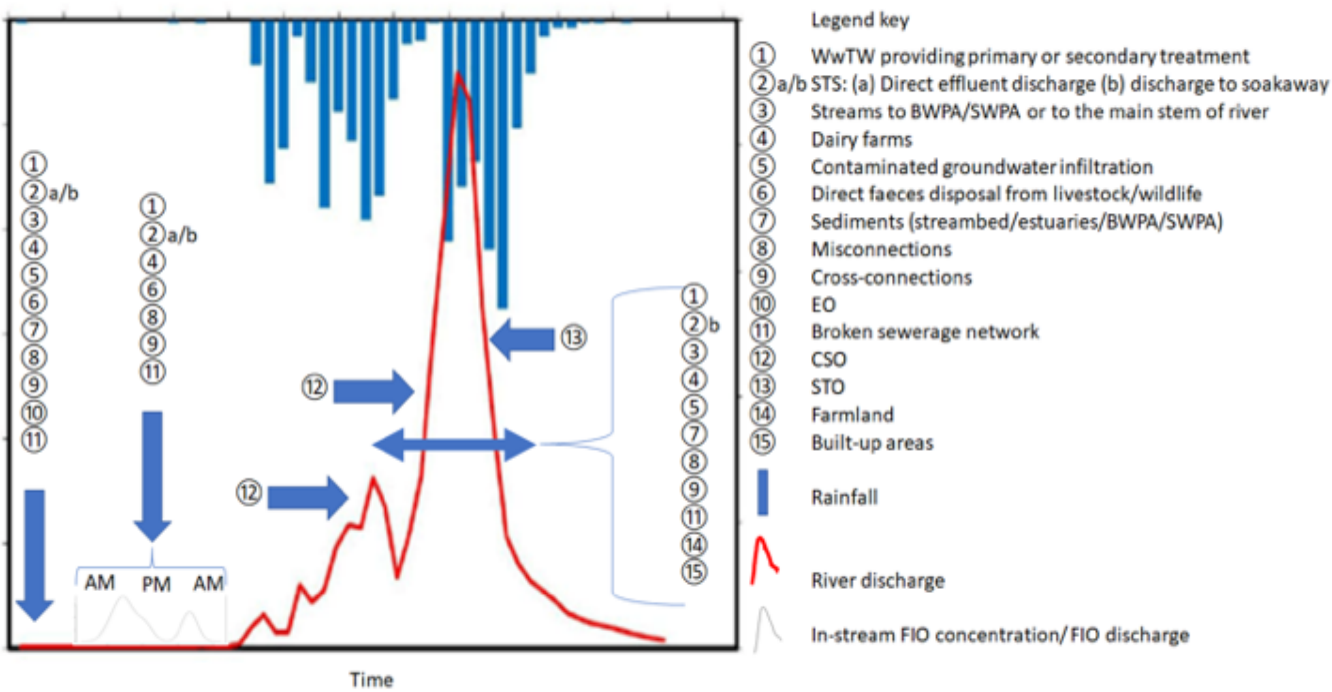

Figure 6. Visualisation of daily FIO variability in discharges and in-stream concentrations and timing of FIO discharges during baseflow and stormflow.

It should be noted that our recommendations regarding the choice of monitoring technologies are based on the requirements from SEPA; this does not preclude the use of the technologies that were not recommended for other purposes.

- **Emerging technology.** We recommend the use of aquaCHECK365 by Brightwater Diagnostics. This device is similar to the Colitag mobile lab and comprises of a built-in incubator and UV lamp. Enumerating FIO is based on growth in selective media. This technology is easy to use and can be used by a non-expert on site, therefore it is useful for remote sites. It comes to market in 2019.

4.2 CREW project team's recommendations for Phase 2 of the monitoring strategy

- **Monitoring design.** We recommend that all approaches must be considered when deciding temporal monitoring, depending on types of sources and their proximity to the coast and watercourses. Generally, we recommend:
  - Year-round monitoring for the weekly, monthly, seasonal and hybrid approaches.
  - Concurrent monitoring of FIO, temperature and flow, regardless of whether hourly, weekly, monthly, seasonal event-based monitoring or hybrid is used.
  - Hourly monitoring for a day or two upstream and downstream of continuous human (e.g. secondary WwTW and STS clusters) and/or animal (e.g. livestock farmland) FIO discharges. This sampling can be performed in wet and dry days for comparison.
  - Weekly or hybrid monitoring in river networks draining to BWPA and SWPA with persistent FIO pollution issues. This monitoring can be established downstream for rainfall-dependent FIO sources, at stream-river confluence sites and at BWPA and SWPA.
- **Current technologies**. We recommend the use of membrane filtration methods and the Colitag mobile lab by Palintest (Appendix IV). Current membrane filtration techniques remain the best current methods for measuring FIO, provided that an appropriate medium is selected. The reasons for recommending Colitag mobile lab are given in Section 3.2.1.3.
- **Emerging technologies:** We recommend the use of Flow cytometry and ALERT – *E. coli* Analyser (Fluidion Range) by Planet Ocean Ltd (Appendix IV). Flow cytometry is recommended for its potential (see Section 3.3.2.1). The ALERT – *E. coli* Analyser can be used at remote sites and can be triggered remotely using a mobile phone, allowing for continuous FIO measurements. It can make seven measurements on a single battery charge. It is also easy to use requiring little training. The ALERT – *E. coli* Analyser could also be used to measure other water quality parameters including pH, chlorine, phosphates and nitrates.

4.3 CREW project team's recommendations for Phase 3 of the monitoring strategy

- **Monitoring design.** We recommend applying the hybrid monitoring design to understand predominant FIO sources from CSO, waterbody catchments, STS clusters or rural residential areas, and at BWPA and SWPA.
- **Current technologies.** We recommend the use of QPCR of genetic markers. This is because QPCR remains the best microbial source tracking tool. It is also the least labour-intensive of the molecular methods (Appendix IV), therefore, results could be obtained within a shorter timeframe. However, care must be taken when choosing primers to amplify genetic markers, as primers are not always 100% specific).
- **Emerging technologies.** We recommend the use of microarray and flow cytometry. The microarray allows high throughput QPCR amplifying several markers (potentially hundreds) simultaneously in a single run, thereby adopting the recommended toolbox approach for microbial source tracking (see Box 4). More work is required to develop this as sensitivity is currently very low. Flow cytometry is once again recommended in this section due to its potential to be adapted for FIO. If successfully adapted for this purpose, it would be the least labour-intensive method and results could be obtained in 1.5h.

5.0 Concluding remarks

Developing meaningful monitoring programs for managing microbiological water quality in BWPA and SWPA requires understanding of the factors influencing transport of faecal microorganisms from sources to BWPA and SWPA. Here, we reviewed available evidence on the factors determining faecal microbiological pollution across a river catchment. We also outlined strategies about how, how often and where to collect microbiological samples in-stream to identify sources of faecal pollution to BWPA and SWPA and to assess contribution from different types of faecal sources (i.e. animal versus human). To address the objectives of this project, we undertook a literature review, developed a GIS-based approach to detecting potential FIO hotspots and developed recommendations for a practical monitoring strategy to detect the area of influence to BWPA and SWPA and track FIO from different types of sources. This helped to develop recommendations for a FIO monitoring strategy for Scotland.

The key findings are summarised below.

- There is sufficient understanding of the broad factors determining timing of FIO discharges from different types of sources, in-stream FIO variation and FIO pollution risk across a river catchment.
- There is consensus among experts on the monitoring strategy needed to identify the area of influence and FIO hotspots therein as well as to differentiate between types of sources (i.e. human vs animal) within the area of influence.
- Current FIO technologies successfully used for FIO catchment investigations in the lab include cultivation-based methods (e.g. membrane filtration, Coliscan Easy gel system and Colilert); DNA-based methods (e.g. qPCR); biomarkers (e.g. sterols); or chemical tracers (e.g. caffeine, saccharin).
- Current FIO technologies successfully used for FIO catchment investigations in the field include using mobile labs after sample collection (without storage time) (e.g. Aquaflex and Colitag) and probes for proxy measurements (e.g. turbidity, conductivity, ammonia and temperature), or using *in situ* devices for continuous measurements (autosamplers).
- There is limited published information for the use of emerging FIO technologies in FIO catchment investigations. Technologies that could possibly be applied include:
    - o In the lab after field sample collection (e.g. RNA biosensors, Flow cytometry and Fluorescent Activated Cell Sorting, Paper-Origami DNA microfluidics and DNA-based methods for microbial source tracking (MST) such as microarray).
    - o In the field, probes (e.g. Bacti-Wader, aquaCHECK365, Bactiquant Water, Microbial Bioanalyser), or continuous monitoring technologies based on the detection of enzymatic activities (e.g. BACTcontrol).
    - o Emerging technologies are most powerful when used in combination with current technologies (e.g. aquaCHECK365 applied in combination with Colitag or turbidity sampling).
- Frequency of sampling for a given current or emerging FIO technology depends on the purpose of sampling and knowledge of in-stream FIO variability at different scales at a given site and time. However, sampling frequency per FIO technology remains briefly addressed in the literature.
- The monitoring strategy to detecting catchment-based FIO sources involves three phases:
    - o Phase 1 identifies the area of influence and FIO hotspots therein through field surveys and monitoring with a desk-based initial screening component in data-rich catchments.
    - o Phase 2 studies in-stream FIO variability in relation to rainfall-dependent/-independent discharges from FIO hotspots in the area of influence through monitoring and modelling.
    - o Phase 3 involves monitoring in the area of influence to elucidate predominant types (i.e. human vs animals) of diffuse FIO sources using microbial and chemical source tracking tools.
- FIO discharges may be rainfall-dependent (e.g. CSO, STO and farmland runoff) or rainfall-independent (e.g. WwTW and STS effluent, artificial drains, livestock, wildlife, and leaching from STS soakaways).
- Temporal variability of in-stream FIO concentrations may be diurnal, storm event-scale, seasonal and interannual.

Our recommendations on where, when, and how to sample to detect FIO are summarised below.

Phase 1: Apply a toolbox approach integrating desktop studies, field monitoring and modelling:

1. Use the desktop screening approach developed here to identify potential FIO hotspots, e.g.:

- Point sources such as CSO, STO, WwTW serving more than 5000 people or tourist resorts; high-density STS clusters (>20 STS/km^2) and STS within 10 to 50m from watercourses located on soils at high runoff risk/ leaching potential.
- Diffuse sources including modelled areas of high in-stream FIO risk from livestock.

2. Apply mobile lab technologies such as Colitag and aquaCHECK365 in combination with turbidity[15], temperature and flow to verify locations and FIO pollution from each potential FIO hotspot identified in the desktop study, as follows:

- Start from the waterbody catchments adjacent to BWPA or SWPA (i.e. coastal catchments).

15 For detecting wastewater downstream of point sources and not as a surrogate for FIO, unless preliminary data suggest that turbidity correlates significantly with levels of FIO in the study area.

- Prioritise human FIO hotspots (i.e. CSO, WwTW, STO, STS) or stream-river confluence sites draining areas influenced by human FIO sources in the coastal waterbody catchments.
- Inspect area for FIO risk from unmapped STS, wildlife, pets and other diffuse sources (e.g. streambed and streambanks) and verify their inputs.
- Select sampling sites that clearly link to known FIO sources, are wildlife-free when sampling, and display small variability during baseflow.
- Collect samples in short periods of time during wet and dry conditions (hybrid monitoring design) to address variability from rainfall-dependent and rainfall-independent discharges.

3. Identify the upstream limit of FIO pollution through monitoring upstream and downstream ("bracketing") potential FIO hotspots until a "clean" sample indicates no FIO impact from upstream. The area of influence may be sought upstream from the coastal waterbody catchments.

Phase 2: Apply membrane filtration techniques and flow cytometry in the lab or use mobile labs (e.g. Colitag) or continuous monitoring devices (e.g. ALERT – *E. coli* Analyser) to assess temporal variability of in-stream FIO (area of influence) concurrently with turbidity, temperature and flow.

Monitoring can be:

- Hourly for a day or two upstream and downstream of continuous human (e.g. WwTW and STS clusters) and/or animal (e.g. livestock farmland) FIO discharges during wet and dry days.
- Weekly or twice weekly (bi-weekly) for as long as necessary to understand discharges from CSO, STS clusters, and stream-river confluence sites.
- Event-scale to study the effects of rainfall-dependent FIO discharges such as CSO, STO and farmland runoff. Event-scale data can be redrawn from weekly time series.

Phase 3: Apply microarray, qPCR of genetic markers or flow cytometry for MST to track predominant FIO sources at sites influenced by diffuse FIO sources or mixed land use. This sampling is confirmatory or hypothesis-driven based on the evidence from Phase 1 and 2 on in-stream FIO variability downstream of CSO, STS clusters, confluence sites and at BWPA/SWPA. Sampling for MST can target wet and dry conditions or be one-off.

We believe that the findings of this project and our recommendations will substantially help SEPA to develop a practical blitz FIO monitoring programme.

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