Environment International 64 (2014) 124–128

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Opportunities and limitations of molecular methods for quantifying microbial compliance parameters in EU bathing waters

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ARTICLE INFO

Article history:
Received 9 November 2013
Accepted 18 December 2013
Available online 4 January 2014

Keywords:
Epidemiology
EU Bathing Water Directive
Faecal indicator organism
Microbial pollution
qPCR
Recreational water

ABSTRACT

The debate over the suitability of molecular biological methods for the enumeration of regulatory microbial parameters (e.g. Faecal Indicator Organisms [FIOs]) in bathing waters versus the use of traditional culture-based methods is of current interest to regulators and the science community. Culture-based methods require a 24–48 hour turn-around time from receipt at the laboratory to reporting, whilst quantitative molecular tools provide a more rapid assay (approximately 2–3 h). Traditional culturing methods are therefore often viewed as slow and 'out-dated', although they still deliver an internationally 'accepted' evidence-base. In contrast, molecular tools have the potential for rapid analysis and their operational utility and associated limitations and uncertainties should be assessed in light of their use for regulatory monitoring. Here we report on the recommendations from a series of international workshops, chaired by a UK Working Group (WG) comprised of scientists, regulators, policy makers and other stakeholders, which explored and interrogated both molecular (principally quantitative polymerase chain reaction [qPCR]) and culture-based tools for FIO monitoring under the European Bathing Water Directive. Through detailed analysis of policy implications, regulatory barriers, stakeholder engagement, and the needs of the end-user, the WG identified a series of key concerns that require critical appraisal before a potential shift from culture-based approaches to the employment of molecular biological methods for bathing water regulation could be justified.

1. The debate

The EU Bathing Water Directive (BWD) 76/160/EEC (CEC, 1976) engages stakeholder interest because of its impact on tourism, local

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http://dx.doi.org/10.1016/j.envint.2013.12.016

economies and public health, and is well publicised through beach award schemes (Guimaraes et al., 2012). However, it also generates controversy across the scientific, regulatory and policy communities with regular debates being driven by scepticism of whether: (i) *Escherichia coli* is a suitable faecal indicator organism (FIO) to assess recent faecal pollution (Wu et al., 2011), (ii) the Directive is suitably protective of human health (Kay et al., 2004; Langford et al., 2000), and, more recently, (iii) the methods currently used to determine microbial water quality at bathing beaches are fit for purpose (Oliver et al., 2010).

These debates are healthy and, as is often the case, more questions are raised than definitive answers provided. However, what we do know is that from 2015 the number of EU designated bathing waters falling below the legally enforceable 'sufficient' standard (equivalent to a 90 percentile of >185 CFU/100 mL and >500 CFU/100 mL of intestinal enterococci and *E. coli*, respectively) could limit the use of EU bathing waters if the non-compliance continues beyond 2020 when the 2006 revised Bathing Waters Directive (rBWD) 2006/7/EC (CEU, 2006) in Europe takes full effect.

The enforcement of the revised BWD in Europe is likely to encourage member states to further improve wastewater infrastructure, and promote better integrated catchment management, as well as provide a significant impetus for the environmental regulators responsible for protecting our bathing waters as 'protected areas' as defined in Annex 4 of the Water Framework Directive (CEC, 2000) in Europe. This immediate focus, however, detracts attention from a more subtle, yet equally complex debate centred on the use of molecular biological testing and the transition of molecular methods from predominantly research tools to standardised protocols for evaluating water quality at bathing waters (Gooch-Moore et al., 2011; Griffith and Weisberg, 2011; Nevers et al., 2013). Current culture-based methods used to enumerate FIOs require a 24–48 hour turn-around time from receipt at the laboratory to reporting, whilst quantitative molecular tools provide a more rapid assay (approximately 2–3 h). Traditional culturing methods are therefore often viewed as slow and 'out-dated', although they still deliver an internationally 'accepted' evidence-base. In contrast, molecular tools have the potential for rapid analysis although they are not yet established enough in the EU for regulatory monitoring.

However, it is important to note that microbial water quality testing at designated bathing waters in the EU can serve two separate purposes. The first is the provision of a monitoring framework for reporting and regulation of microbial water quality and the second is in helping control the public health risk from microbiological contamination of bathing waters. The first purpose is effectively 'state of the environment' monitoring to collect sufficient data to produce information on general status of bathing water quality and infer how well our management practices and policies are working, and whether environmental outcomes are being achieved. This data is collected over the longer term and can be summarised into a bathing water classification and may contribute to a beach award. The second purpose is about assessing the risk of an individual bathing event. Thus, the time delay of culture-based approaches leads some scientists to question whether rapid molecular methods could play a more effective role in assessing the risk of individual bathing events. This is a debate that is international in scope, but which was driven principally by the need for new recreational water quality criteria in the US. The US movement was prompted by a lawsuit against the US Environmental Protection Agency (USEPA) filed by the Natural Resources Defence Council (NRDC) which argued that the USEPA had not delivered on its intention to explore new or revised water quality criteria linked to 'rapid test methods' (Gooch-Moore et al., 2011). This led to the publication of revised standards based on the voluntary use of molecular biological methods, principally quantitative polymerase chain reaction (qPCR) analyses. Thus, the crux of the debate centres on the relevance and effectiveness of existing (culture-based) methods compared with promising (qPCR-based) quantification methods for enumerating microbial compliance parameters at designated bathing waters and whether either relates to human health risk.

If, in time, qPCR is adopted widely in the US as a method of choice for quantifying levels of faecal pollution then pressure may begin to build in the UK and the rest of Europe to follow suit for enumerating these regulatory microbial parameters within the EU Directives (Oliver et al., 2010). In response, a Working Group (WG) was established in the UK, under the auspices of the 'Delivering Healthy Water' project. The WG drew on international expertise via a series of workshops to debate the utility of qPCR methods versus culture-based approaches for microbial water quality analysis linked to regulatory monitoring. The overarching aims of the WG were to: (i) interrogate the existing evidence-base and (ii) provide a balanced evaluation of the associated uncertainties, benefits and limitations surrounding such a shift in methodological approach for bathing water monitoring and regulation.

2. From research tool to standardised protocol: five hurdles to overcome

The WG identified a series of key recommendations needed to underpin adoption of the new molecular biological methods by regulatory bodies. These reflect generic scientific considerations but focus the lens of debate on a European policy perspective. Each recommendation is dealt with in the sections below.

2.1. Recommendation 1: building the epidemiological evidence-base

Demonstrating a robust relationship between (a) molecular marker(s) and human health outcomes (i.e. infection or illness in bathers) via an epidemiological evidence base is of fundamental importance before any shift from a culture-based to a qPCR-based approach can be considered across the EU. This priority recommendation was also identified by a group of international experts convened to debate the transitioning of new methods from research and development to an operational phase as part of the US recreational water quality criteria (Boehm et al., 2009). Recent epidemiological studies in the US have explored the relationship between FIO concentrations and gastrointestinal infections using qPCR methods (Wade et al. 2006, 2010), however, these studies focus only on beaches impacted by human sewage and consequently their generic relevance to bathing waters in Europe (which are more likely to be impacted from diffuse sources) is unclear.

It is critical that we understand how transferable the dose–response relationships from epidemiological studies at locations dominated by point sources are, particularly when differences between the risks associated with human and ruminant wastes are so poorly characterised (Boehm et al., 2009; Dufour et al., 2012; Gooch-Moore et al., 2011; Till et al., 2008) and the relationship between levels of exposure and incidence of illness in the wider population fraught with unknowns (Bridge et al., 2010; Soller et al., 2010). Others have begun to investigate the role of qPCR versus culture in sub/tropical diffuse source recreational marine waters and proposed further epidemiological studies in order to explore possible dose–response relationships between human illness with indicator organisms (Sinigalliano et al., 2010). We advocate the need for a series of robust international epidemiological studies that span a number of European bathing water types that are impacted by point sources (e.g. sewage contributions), diffuse source inputs, and sites that experience a mix of both sewage-derived and diffuse source contributions to the overall microbial load. We also argue that it would be essential to undertake such epidemiological studies by measuring culture and qPCR-based targets in parallel and in the same sample to provide a definitive back-to-back comparison of the methods across a suite of international waters. The provision of a cross-comparison data set derived using both culture based and molecular methods to quantify microbial parameters would allow for some exploration of parity to historical data sets. In time, these studies

would need to complement the development of threshold doses for regulators to use in compliance monitoring of bathing waters.

2.2. Recommendation 2: establishing accuracy and precision

An advantage of molecular tools over culture-based approaches is undoubtedly their specificity and sensitivity. The specificity of qPCR is often promoted as a reason for using it as a tool to quantify specific pathogens, which would avoid the paradox of using FIOs as surrogates for the presence of a wide range of viral, bacterial and protozoan pathogens (Quilliam et al., 2011). However, this needs to be set against a backdrop of uncertainty surrounding the general consensus amongst the research and regulatory communities over what constitutes the best pathogen(s) to target. Pathogen enumeration is, of course, a very different issue to address given that their presence/absence can be highly episodic; although absence indicates no risk of that infection at that point in time, or at that specific location, it does not confer or imply protection outside of this defined spatial–temporal relationship.

Any analytical approach must be underpinned with certainty that the data exhibits clearly defined (accurate) and reproducible (precise) results based on international inter-laboratory ring trials, i.e. they give a true representation of the parameter being measured within a defined and acceptable level of confidence. Therefore, the use of qPCR for bathing water analysis has some significant hurdles to overcome before any potential widespread transition from research tool to standardised protocol. Site specific feasibility studies are warranted to determine whether qPCR approaches are suitable for particular locations given the occurrence of analytical inhibition resulting from the complex nature of environmental matrices (Nevers et al., 2013). This is perhaps especially true given the observation that the qPCR signal from commonly used microbial source tracking (MST) markers seems unaffected by sewage treatment processes such as UV disinfection (Stapleton et al., 2009). However, results from the US are contradictory with studies reporting comparable reductions in viable cells and qPCR calibrated cell equivalents following UV treatment (Kinzelman et al., 2011; Lavender and Kinzelman, 2009). Until such conflicting evidence can be sufficiently explained, and controlled for, it will pose a significant barrier to wider implementation of qPCR as a regulatory tool for bathing water quality assessment in the EU.

Reproducible results determined across multiple laboratories are also critical: the same sample processed at different laboratories should in theory result in consistent reporting. Unfortunately, the reality falls short of this theoretical ideal, and there is evidence of significant variability (~one order of magnitude) being reported in qPCR data obtained from different investigators using the same approaches (Shanks et al., 2012). Inter-laboratory studies tend to use professional research laboratories in their ring-trials and will typically use experienced staff (Shanks et al., 2012). However, the wider roll-out of qPCR protocols to less proficient laboratories and the challenge of ensuring technology transfer to personnel who may have little molecular biology experience, are likely to result in significant data variability, and could deliver less reliable results (Noble et al., 2010). High quality and continuous training would therefore be a prerequisite to ensure that staff understood fully the breadth of potential sources of variability in qPCR methods and results.

Furthermore, there is evidence that replicated qPCR estimates from a single sample can have a relative error that exceeds that observed in replicated culture counts even at relatively high target levels (Whitman et al., 2010). Moreover, a smaller volume of bathing water sample can be analysed questioning representativeness. And in that respect reduction of inhibition versus testing sufficient sample volume is under debate (Rutjes et al., 2006). Considerable investment would also be needed to ensure standardisation of the preferred approach and protocol interpretation, although we acknowledge that this would be a problematic barrier to overcome given the difficulties in securing funding for technology development. Concerns over the lack of method standardisation (often related to method complexity and lack of researcher consensus over protocols) have been reported elsewhere (Girones et al., 2010), leading regulators to express concern that any shortcomings in accuracy and precision, whether real or perceived, could render data obtained by such methods inappropriate for use in legal proceedings.

2.3. Recommendation 3: consider rapidity & logistics — how fast is fast enough?

Molecular methods such as qPCR offer a much faster analysis time than culture-based methods, e.g. 2–3 h compared to 24–48 h (Griffith et al., 2009), but it is necessary to consider the amount of practical benefit achievable from the increased speed in sample turn-around time. For example, any bathing water sample collected from a designated site in England is transferred to a centralised regulatory testing laboratory in the southwest of the country. Therefore, a sample from the northwest or northeast of England will incur an overnight transfer from the beach to the laboratory before the analysis can be undertaken. This issue is transferable to other EU member states that process samples at a centralised laboratory rather than using regional or local facilities. Thus, the adoption of qPCR because of its capability to deliver rapid results can be affected by governance structure and centralised laboratory infrastructure.

Establishing regional laboratories to facilitate more rapid analysis and sample turn-around times would require considerable shifts in existing infrastructure, and would reinforce rather than abate earlier concerns regarding potential for inconsistencies in qPCR reporting (see the Recommendation 2: establishing accuracy and precision section). Whilst this may limit the application of qPCR as a regulatory tool it is still important to consider its potential, not least because a number of stakeholder communities are interested in how they may be able to receive a more immediate, 'real-time', statement of the risk posed by bathing water quality in order to make better informed decisions. The argument for speed is only valid if such an approach is used regularly (i.e. daily) as there is little value in knowing quickly about bathing water quality if sampling is only undertaken once a week. This argument leads to two further concerns: (i) samples taken in the morning and analysed using qPCR may not characterise the variability of microbial pollution that may occur throughout the bathing day (Boehm, 2007; Boehm et al., 2002; Mudd et al., 2012) and therefore the need for speed is, in such cases, redundant; and (ii) issues of cost and available resources make daily sampling prohibitive, although arguably even daily sampling is not frequent enough.

It is generally well accepted that rapid methods such as qPCR do offer exciting opportunities in the broader context of catchment 'forensics' and MST for exploring upstream pollution sources, particularly when used as one component of a wider 'toolbox of methods' (Abdelzaher et al., 2013; Santo Domingo et al., 2007; Staley et al., 2012; Stapleton et al., 2009). It is important therefore, to recognise that part of this methodological debate linked to regulatory monitoring is hampered by the fact that the Directives do not seek to understand sources, pathways and time-scales of FIO transfers. Instead they form an end-point procedure, and this equates to a fundamental difference in requirements between regulator and end-user.

2.4. Recommendation 4: identifying value for money

The economic considerations associated with method transition are complex and extend far beyond the costs of the capital outlay and the consumables associated with culture versus qPCR-based approaches (Griffith and Weisberg, 2011). Even at this rather simplistic level of accounting for costs, the transfer from culture to a molecular approach could not proceed seamlessly without an initial phase of concurrent monitoring and analysis via both culture and qPCR, which would

involve significant resource implications at a time when finances available for environmental protection are limited.

However, there are a multitude of wider economic debates linked to indirect costs of method transition that have received little, if any, attention in previous assessments of the culture to molecular transition (Rabinovici et al., 2004). Economic assessments of moving from the 1976 BWD to 2006 rBWD (e.g. Georgiou and Bateman, 2005; Hanley et al., 2003) provide a useful template for the exploration of wider economic implications that may arise from any future protocol changes within the rBWD. Amongst these are considerations of how changes to beach and bathing water use would take shape (e.g. frequency of visits and activities) should water quality information be improved in terms of speed of provision to the beach-user community. Other key questions relate to how qPCR-related classifications might affect tourism at coastal resorts and the associated willingness of the public to pay for receiving rapid water quality information.

Perhaps the most important of all the 'value' related questions are those surrounding the types of information beach users actually require; how quickly they need it; and how it is best disseminated. In response we argue that *prediction* of bathing water quality could have far more *value* to beach users than 'real' water quality data that is, by its very nature, always out of date by the time it is communicated to the public i.e. people want to know what the risks are before they enter the water. Others have also stressed the potential value of modelling (Kay et al., 2008; Nevers et al., 2013; Oliver et al., 2009; Shibata et al., 2010). Whilst the development of models to predict health risks will be inherently 'data hungry' for culture-based counts and therefore not necessarily cheap, such models developed using culture-based methods could actually provide a far more cost-effective 'rapid method' for delivering information on water quality. Consequently, predictive models could offer a significantly reduced investment relative to wastewater infrastructure upgrades in terms of managing risk.

2.5. Recommendation 5: establishing time frames for implementation

Embedding a new method into legislation can take considerable time, and there needs to be sufficient underpinning evidence to support its inclusion in revisions to any Directive. An awareness of policy reviews, associated timescales, and the opportunities to feed into government consultation are therefore essential if new approaches are to eventually garner favour amongst both the science and regulatory communities and the transition from research tool to standardised protocol is to be realised. Coupled with this is the need for programmes that raise awareness with beach and bathing water users to ensure efficient and clear communication about the nature of any changes and their interpretation. Within the EU the next review of the rBWD is scheduled for 2020 but given the challenges outlined above this could prove to be a testing timeframe for settling all of the debates over the opportunities and costs of molecular biological tools for bathing water compliance monitoring.

3. Tides of change

Molecular biological testing offers new opportunities over culture-based methods not least with respect to near real-time reporting on bathing water quality. However, the current requirements of the rBWD are for compliance records to be maintained and for this the speed of response is not a priority for regulators. Beach users are likely to disagree and of course qPCR may offer value in providing a more rapid response for bathing water 'advisory' notices following known pollution events. Ultimately the most useful 'rapid method' may perhaps be found just outside of the laboratory in the form of modelling and forecasting tools that allow regulators to understand what the predictable risks to bathing water quality are so that in turn they can then begin to manage those risks. Laboratory assessments and analytical techniques are implicitly linked to the development of those models but the future of rapid methods may not necessarily be of a molecular biological nature. Instead 'value' in its widest sense might be best found in trying to predict risks to human health. Crucially, we need intensive data sets to underpin model development and testing; therefore predictive capability is certainly not a 'quick fix'. However, by managing expectations of different beach user groups, reinterpreting what we mean by rapid methods, shifting focus to prediction underpinned by quality data and communicating the limitations as well as perceived benefits of molecular capability to the policy community we should be confident that the tides of bathing water regulation will continue to change for the better.

Acknowledgements

The Working Group and associated workshop series were funded by the Natural Environment Research Council as part of the Delivering Healthy Water project (NE/I022191/1). LF received funding in part from the European Regional Development Fund Programme and the European Social Fund Convergence Programme for Cornwall and the Isles of Scilly. The authors acknowledge the valuable contributions made by Tim Wade at the workshops in London and the constructive comments of the anonymous referees.

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