Drink. Water Eng. Sci. Discuss., 5, C294–C305, 2012
www.drink-water-eng-sci-discuss.net/5/C294/2012/

Drinking Water Engineering and Science Discussions

Interactive comment on "Application of DVC-FISH method in tracking *Escherichia coli* in drinking water distribution networks" *by* L. Mezule et al.

L. Mezule et al.

linda.mezule@rtu.lv

Received and published: 24 December 2012

We thank Referee #2 for the comments.

In general I think further detailed discussion is required on the importance of the detection of VBNC cells in the context of whether it a) makes culture data meaningless, b) whether the wrong indicator is being chosen, c) is this important as no outbreaks have been recorded within the study timeframe? There are many studies which have recognized the presence of VBNC bacteria in such an environment and what additional information does this study bring? I think the authors need to present stronger arguments for their ïňĄndings. In general there needs to be improvement on the writing style and use of English as several sections are confusing to read. In addition, there have been little statistical analyses carried out and rarely are data quoted in the text.

The comments are taken into account by extending the discussion in Results and Discussion part of the paper to cover 3 issues raised by the reviewer. However, it is clear that more studies are needed to address these problems for explicitly.

SpeciïňĄc comments: Abstract: P516, L6 "analyse the distribution of non-cultivable E. coli" – important to clarify this statement with non-cultivable but viable, there is no point in just detecting non-cultivable.

Here in this study we aimed to basically distinguish between those E. coli which can be grown to a visible colony and those that cannot. The term "viable" was not inserted into this sentence because we were not distinguishing between metabolically active, enzymatically active E. coli or membrane integrity. Our aim was to determine E. coli in distribution systems as such. To analyze viability we used DVC method were cell ability to divide is determined. This is a close term to cultivability, however, first not all cultivation methods will give the same results so on some media there might be no colonies. Secondly, if a bacteria is able to divide it does not mean that it will be able to form a colony.

P516, L12 "cultivable, total and able to divide (viable)" – throughout the manuscript there are discrepancies on how the term "viable" is used. In this instance, it is confusing because cultivable bacteria are also able to divide and are obviously viable. More clariïňĄcation of deïňĄnitions is needed.

Cultivable – those that form colonies or any other visible marker detectable by traditional methods. Total – all E. coli cells. Able to divide (viable) – those cells that show potential for dividing (maybe not able to form a colony) – can divide at least once.

P516, L17 "almost all of the samples contained viable E. coli in the range of 1 – 50 cells per litre or cm2 which represented approx. 53% of all E. coli detected." – this sentence needs clariïňĄcation, how have these 53% been detected? Does viable include those which are cultivable? And how has total E. coli (including non-viable) been calculated? There is no information on these points and although only the Abstract it makes it

difïňĄcult for the reader to understand the approach.

As described previously we determined able to divide (viable) E. coli with DVC-FISH method – these were these 53%. Total E. coli concentration was determined as total FISH stained E. coli. The difference between total and viable was determined according to description from Bjergbaek et al – those bacteria which have a potential for dividing form elongated (more than 1.5 mkm long) cells after incubation with nutrients (elongation determined with ImagePro Plus software) but those that have not – remain in their standard size ($\sim$ 1 mkm for environmental, starved or stationary phase E. coli, internal data). A part of these viable (DVC-FISH positive) E. coli could be cultivable, however, our simultaneous cultivability analyses did not showed any cultivable cells at al in the biofilm. Bjergbaek, L. A. and Roslev, P.: Formation of nonculturable Escherichia coli in drinking water, J. Appl. Microbiol., 99, 1090–1098, 2005.

P516, L18-20 – a comment is made that the number of viable E. coli tend to increase further from the treatment plant but no explanation offered. However, in the following sentence it is stated that it accumulates when less exposed to disinfectants – this is an obvious statement and these sentences could be condensed and combined.

Taken into account.

P516, L23-25 – the term “viable but not cultivable” is used for the ïňĄrst time, this should be used in the earlier deïňĄnitions.

Taken into account.

General point – what is the clear conclusion of the work, what new information does it reveal? If the focus is to be how DVC-FISH is an improved method, this should be stated in the abstract.

Abstract was modified to more emphasise the focus of the study.

Introduction: P517, L17 – the authors state that it remains disputable whether the VBNC state exists but there is now an extensive amount of more recent literature

demonstrating the existence.

The amount of studies indicating on the possible existence of VBNC is indeed extensive. However, in most cases this is made as a result of observations or analyses showing that there are cells that cannot be grown on culture media, however, have the same gene expression (most often down-regulated). To our knowledge there is no clear study describing specific genetic activity occurring only in VBNC state bacteria.

P517, L20-25 – comments are made regarding the retention of pathogenicity but no mention of infectivity studies on VBNC bacteria in mice, rabbits, embryos and amoebae (amongst others) is made.

These studies usually employ the use of large feeding doses (even VBNC) of cultures obtained in laboratory. However, this is not the scope and idea for this study where the concentrations of even non-culturable E. coli detected are relatively low. The only exception is the potential occurrence of pathogens and their toxins in water or biofilm. Additionally, it has been proven that VBNC E. coli (pathogenic form) can generate Shiga-like toxins. The reference to Pommepuy et al. will be changed to a more novel reference on toxin formation in VBNC E. coli. Liu Y., Wang C., Tyrrell G., Li X F.: Production of Shiga-like toxins in viable but nonculturable Escherichia coli O157:H7, Water Research, 44, 711-718, 2010

P518, L7 – the authors refer to detecting total, cultivable and VBNC (detected as DVC-FISH) cells, however, care must be taken to acknowledge that DVC-FISH will detect cultivable plus VBNC. The number of VBNC can be calculated by subtracting cultivable values from DVC-FISH results.

A change will be made and the term “VBNC” will be changed to “potentially dividing” (P.518, L7). The scope in this study was to determine E. coli cells with the potential for dividing. To determine true VBNC additional viability markers should be used – like membrane potential, enzyme activity etc. In our case DVC-FISH negative, FISH positive cells still could have metabolic activity. However, since E. coli concentrations

in water are generally low and infectious dose rather high (with exception of certain pathogenic strains), completely non able to divide cells should not pose the threat, with the exception if the concentration is very high. The latter can be estimated by general FISH analyses or detection of DVC-FISH negative cell proportion.

Methods: In general it is noted that some sections of the methods are given in detail (e.g. composition of hybridization buffer) and other sections are very vague. P518, L20 –a reference from 2010 is given related to water quality but no acknowledgement is made that this can change within a short time frame and within a network and so the referenced data may not be applicable.

This reference is supposed to give explanatory information on general water quality and preparation technologies used – that the water is generally rich in natural organic matter, etc., not a specific report on everyday water quality. According to local legislation the water is prepared so that the quality corresponds to Drinking Water Directive 98/83/EC.

P519, L5 – the bioïňĄlms were tested after 2 weeks, this does not seem a very long period to allow bioïňĄlm development and can this really be considered to be comparableto long term bioïňĄlm which will have built up over years? Some discussion is needed in the later sections.

As reported to the comments to Referee #1 (F. Hammes): Two week long biofilm growth was selected based on previous experience – this time was selected as the shortest period allowing to obtain recordable and more or less stable biofilm. Long term incubation (more than 1 year) was not evaluated due to simultaneous accumulation of organic and inorganic matter. The studies on determination whether the detected E. coli come from accumulation or growth are performed with our research group at the moment. P519, L12 – the water concentration step is noted as taking between 12 and 24 hrs, arethe samples kept at a constant temperature through this time period? Is there evidence to show that differences in concentration time do not affect results? The

concentration step was performed in situ at water concentration site (treatment station, pumping station or the site of biofilm collectors) the time varied depending on the quality of water collected – shorter concentration time was chosen for untreated waters to overcome problems of membrane clogging etc. According to publication by Veenendaal and Brouwer-Hanzens (2007) the recommended maximum volume of sample undergoing concentration was 2000 l. This was chosen as a reference and concentration time was determined based on this issue. Veenendaal, H. R. and Brouwer-Hanzens, A. J.: A method for the concentration of microbes in large volumes of water in, D 3.2.4, TECHNEAU, 1–30, 2007.

P519, L14 – the recovery efïňĄciency is stated as 8133% , this means in some casesit was as low as 48% or as high as 114%, I feel a comment on this needs to be made as it can clearly affect results considerably.

First of all this in no case affect biofilm studies. Then, this was estimated specifically for E. coli in drinking water. Higher efficiency rates were observed directly after the treatment. More detailed information have been described elsewhere: Veenendaal, H. R. and Brouwer-Hanzens, A. J.: A method for the concentration of microbes in large volumes of water in, D 3.2.4, TECHNEAU, 1–30, 2007.

P519, L18 – the volume of the sample should be stated.

The concentration of water sample was chosen so that the cell count per field of view does not exceed 100 cells (to minimize errors) initially 0.1 ml of sample was filtered, however, if the concentration was too low or too high, an additional sample with the adjusted concentration was filtered. An explanation ... a known volume of sample ($\sim$ 0.1 ml, adjusted to obtain 15 – 100 cells per microscope field of view).

P519, L25-29 – more detail is required to be able to understand the ATP method.

No modifications were made from the original method described by Vital et al. (2008). The detailed description can be obtained in the reference. Additional reference de-

scribing the protocol: Hammes F., Goldschmidt F., Vital M., Wang Y., Egli T. (2010) Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. Water Res 44: 13. 3915-3923

P520, L8-10 – although following a standard method, it would be useful for the reader to have more information on the medium used, incubation conditions, etc.

The analyses were performed in a certified reference laboratory working according to mentioned ISO methodology. Full description is available in these standards. The following explanation will be added into the paper: . . .membrane ïňĄltration method in a certified reference laboratory according to EN ISO 9308-1:2000. . ..

P520, L13 – no details are given on the type of FISH, i.e. DNA or PNA probe? No reference provided for earlier work comparing to molecular methods.

PNA probe was used in this study. This will be included into the paper: . . .The PNA probe (TCA ATG AGC AAA GGT) (Perr y-O'Keefe et al., 2001) was . . .. A reference on the probe sequence has been given. The description of probe work has been given there and the same sequence has been used in previous studies by Juhna et al.

Juhna, T., Birzniece, D., Larsson, S., Zulenkovs, D., Shar ipo, A., Azevedo, N. F., Me'nard-Szczebara, F., Castagnet, S., Fe'liers, C., and Keevil, C. W.: Detection of Escherichia coli in bioïňĄlms from pipe samples and coupons in drinking water distribution networks, Appl. Environ. Microbiol., 73, 7456–7464, 2007 Perry-O'Keefe, H., Stender, H., Broomer, A., Oliveira, K., Coull, J., and Hyldig-Nielsen, J.: Filter-based PNA in situ hybr idization for rapid detection, identiïňĄcation and enumeration of speciïňĄc microorganisms, J. Appl. Microbiol., 90, 180–189, 2001.

P521, L2 – following hybridization the ïňĄlters were washed in distilled water only, usually a washing step using a washing buffer for a certain period of time (e.g. 30 mins) is used to ensure no non-speciïňĄc binding. Could the authors make reference to this change in method or provide evidence of validation?

Typically this step is included in hybridization protocols for oligonucleotide probes. Our previous studies have shown that there is no need for post-hybridization washing in case of this specific PNA probe. The results give high enough fluorescence which is specific to the target organisms. Additional studies indicating this: Mezule L. Significance of Nonculturable Escherichia Coli in Drinking Water: Experimental and Pilot Studies in Large Drinking Water Systems. Lambert Academic Publishing, 2012. Larsson S., Juhna T., Mezule L., Fiksdal L. D 3.5.1. Development of FISH methods for detection of pathogens in biofilm // EC 6th framework project TECHNEAU. - 2007. – pp. 47.

P521, L3 – provide details on microscope used.

On Page 521, line 3 the following explanation will be added: ... microscopy (Ex: 535 $\pm$ 25 nm; Em. 610 $\pm$ 37 nm, dichromatic mirror 565 nm, Leica DM LB).

P521, L4 – how was a detection limit of 4 and 6 cells calculated – was the entire ïňĄlter scanned for labelled E. coli? Normally the multiplication resulting from using a low number of ïňĄelds of view results in a higher detection limit.

The detection limits were calculated based on volume of analysed sample, repetitions and microscope fields counted. In this case 1/5 of the filter was scanned for positive events. The counting technique to obtain these detection limits is described in more detail in: Mezule L.: Significance of Nonculturable Escherichia coli in Drinking Water: Experimental and Pilot Studies in Large Drinking Water Systems, Lambert Academic Publishing, 2012.

Results & Discussion: P522, L13 – it is stated that sampling has been carried out from water source to tap but nowhere in the text is there mention of testing from a tap – could this be clariïňĄed.

The emphasis was made on distribution system. There have been reports from water supplier that the water quality till the house is good and acceptable and all problems

arise from low-quality plumbing systems in the house. We studied the changes of water quality from the station throughout the system and showed that there are differences in water quality depending from the water residence time in the network.

P522, L26/27 – a lowering of detection limits is mentioned but no details are provide comparing detection limits so it is impossible for the reader to assess this. Detection limits (including calculation) are needed for each technique.

The detection limits are mentioned in P521, L3-4. A more detailed description of counting technique is explained and referenced previously (Materials and Methods part). Traditionally microscopy techniques have relatively high detection limits (around 1000 cells per sample volume analysed). We did not employ counting of randomly selected fields but scanning of filter sectors for positive events. Additionally for each type of sample (untreated, treated, different network sites) recovery rates of spiked culture was estimated. The results are not reported in this paper since it was not our aim to propose this technology as a replacement for traditional monitoring techniques but give a more in-depth estimation of the true occurrence of the most popular faecal indicator – E. coli.

P522, L28-30 – it is noted that more cells were found in the spring and winter months (it is not known whether this was statistically signiïňĄcant) suggesting no inïňćuence of warmer external temperatures but it is also stated that the water temperature remains the same regardless of the time of year so why would an effect of outside temperature even be investigated?

The water is taken and treated from surface and groundwater sources, where the temperature might have an effect on the concentration of E. coli. Thus, it was assumed that higher counts should be expected at higher inlet water temperatures. To analyse to effect of seasonality on cell counts in each individual site, single factor ANOVA was performed for FISH and DVC-FISH results obtained. The results showed that for DVC-FISH data there was no significant difference between the values ($p>0.05$) obtained

during different seasons. For the FISH counts significantly different results were obtained only in 2 sampling sites – S-DW ($p<0.02$) and S-NET1 ($p<0.05$) where in winter and spring months respectively higher total E. coli counts were observed. Respectively, for these samples high variance between the repetitions was observed. Some explanations and statistical data will be included in the discussion part of the manuscript.

P523, L17-18 – "existence of naturalized E. coli, although no pathogenicity was proven" – do the authors mean "naturally occurring E. coli"? And they do not show any pathogenicity why should we be concerned?

The main issue is that E. coli is still the most important faecal indicator used for drinking water. Naturally occurring E. coli might have no influence on human health as such, however, if we find these bacteria in water when doing analyses or do not find when they are in water biofilms – we might either underestimate or overestimate the water quality. Moreover – the main concern is if E. coli is an appropriate indicator as such.

P523, L25 – using the terminology of "about million cells" should be clariïňĄed, at least provided in scientiïňĄc notation 106 cells.

A change in the manuscript will be made: . . ...reaching the concentration of more than 106 bacterial cells per cm2 of the biofilm (Table 2). . ..

P523, L26 until end of paragraph – not clear what point is being made, could the authors explain. Also a R2 value of 0.8869 is quite low.

The paragraph explains overall microbiological parameters in the samples analysed for E. coli. We observed that, when in the biofilm the total bacterial counts increase, the cultivable counts increase too. This correlation was not so strong for water (only $R2 = 0.8869$). This emphasizes on the problem when only cultivable counts are analysed for water samples. Additionally, it was observed that microbiological quality for water does not represent biofilm situation – in this case only negative correlation was observed. As a result, if there are no E. coli in water phase, this does not necessarily mean that

biofilm is free from E. coli.

P524, L17 “role of bioïňĄlm community on pathogen accumulation” – this should be explained in more detail as it is not clear in the text how the linear correlation relates to this.

We observed that denser biofilms contained more viable E. coli. This could explain previous observations that mature biofilms better support the survival of pathogenic or non-indigenous microorganisms.

In general, there also needs to be a discussion on the limitations of the DVC-FISH method – is it practical for routine screening, etc? And more discussion on whether existing measures are sufïňĄcient as there has been no outbreaks?

The method itself at the moment employs the use of microscopy counting. At the moment we are not proposing to change the E. coli detection techniques but more emphasizing and describing the problem as such. However, in the future it might be a good tool, since we still analyse bacterial ability to divide (DVC-FISH positive) and as a second parameter (only FISH positive) we get the total concentration of bacteria of interest (as DVC-FISH negative group). For the outbreak situation: as explained in previous comments – a change in the paper will be made by stressing out that there have been no mayor outbreaks. The outbreak registration in Latvia is mostly limited to major outbreaks, which have not occurred. However, there is no information about occurrence of sporadic infections, since relatively mild diarrheal cases are not usually reported. Thus, we can only accept that there have been no cases of infections with pathogenic forms of E. coli, e.g. O157.

Conclusions: The conclusions do not appear to add any new information to the existing knowledge. More emphasis should be made on the effectiveness of the method and a comment on whether existing measures are sufïňĄcient.

The conclusions were modified to better emphasise the progress beyond the state-of-

art, namely, this study for the first time show behavior of different viability forms of E. coli in the water distribution networks in a full scale. It brings about a new understanding of E. coli fate in water distribution system which is important for development of more coherent water treatment and control methods.

Interactive comment on Drink. Water Eng. Sci. Discuss., 5, 515, 2012.