

1      **TaqMan Array Cards enable monitoring of diverse enteric pathogens across**  
2      **environmental and host reservoirs**

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24

25

26 **Abstract**

27 *Background*

28 Multiple bacteria, viruses, protists, and helminths cause enteric infections that greatly impact human  
29 health and wellbeing. These enteropathogens are transmitted via several pathways through human,  
30 animal, and environmental reservoirs. Individual quantitative PCR (qPCR) assays have been extensively  
31 used to detect enteropathogens within these types of samples, whereas the TaqMan Array Card (TAC)  
32 that allows simultaneous detection of multiple enteropathogens has only previously been validated  
33 in human clinical samples.

34 *Methods*

35 Here, we performed a comprehensive double-blinded comparison of the performance of a custom  
36 TAC relative to standard qPCR for the detection of eight enteric targets, by using spiked samples,  
37 wastewater from Melbourne (Australia), and human, animal, and environmental samples from  
38 informal settlements in Suva, Fiji.

39 *Findings*

40 Both methods exhibited high and comparable specificity (TAC: 100%, qPCR: 94%), sensitivity (TAC:  
41 92%; qPCR: 100%), and quantitation accuracy (TAC: 91%; qPCR: 99%) in non-inhibited sample  
42 matrices. PCR inhibitors substantially impacted detection via TAC, though this issue was alleviated by  
43 10-fold sample dilution. Among samples from informal settlements, the two techniques were  
44 comparable for detection (89% agreement) and quantitation ( $R^2 = 0.82$ ). The TAC additionally included  
45 38 other targets, enabling detection of diverse faecal pathogens and extensive environmental  
46 contamination that would be prohibitively labour intensive to assay by standard qPCR.

47 *Interpretation*

48 Overall, the two techniques produce comparable results across diverse sample types, with qPCR  
49 prioritising greater sensitivity and quantitation accuracy, and TAC trading small reductions in these for  
50 a cost-effective larger enteropathogen panel that enables a greater number of enteric pathogens to  
51 be analysed concurrently, which is beneficial given the abundance and variety of enteric pathogens in  
52 environments such as urban informal settlements. The ability to monitor multiple enteric pathogens  
53 across diverse reservoirs in turn allows better resolution of pathogen exposure pathways, and the  
54 design and monitoring of interventions to reduce pathogen load.

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57 **1. Introduction**

58 Diarrhoeal disease due to inadequate sanitation and poor water quality is a major public health issue  
59 and the target of one of the United Nations Sustainable Development Goals (SDG 6). This problem  
60 disproportionately affects lower- and middle-income countries, especially people living in urban  
61 informal settlements.<sup>1,2</sup> Approximately 500,000 children under the age of five die from diarrhoeal  
62 disease each year,<sup>3-5</sup> despite the potential to prevent an estimated 360,000 child deaths by  
63 improvements to water, sanitation and hygiene (WASH).<sup>6</sup> Various nondiarrhoeal pathogens, most  
64 notably helminths, also contribute to enteric disease burden and malnutrition<sup>7</sup>. Moreover,  
65 asymptomatic or subclinical carriage of various enteropathogens also impacts child growth.<sup>8</sup> Recent  
66 evidence has suggested that traditional household level WASH interventions such as pit latrines,  
67 handwashing with soap, and chlorination of water deliver suboptimal reductions in enteric disease in  
68 environments that are densely populated,<sup>9</sup> highly contaminated<sup>10</sup> or have a high prevalence of  
69 diarrhoea.<sup>11</sup> This is likely due to the inability of these interventions to address the many pathways that  
70 connect environmental enteropathogens to community residents. Humans, animals, and their  
71 surrounding environments can serve as extensively interconnected reservoirs for enteropathogens.  
72 Thus, unified 'One Health' and 'Planetary Health' approaches are needed to identify pathogen  
73 exposure pathways and inform interventions that reduce pathogen load in the environment and in  
74 turn reduce human exposure.<sup>12</sup>

75 Assessing the extent of enteropathogen contamination and the impact of new mitigating  
76 interventions in urban informal settlements requires methods that can monitor several  
77 enteropathogen species in a range of sample types. Screening for a large number of enteropathogens  
78 is important, as multiple viruses, bacteria, protists and helminths are responsible for poor  
79 gastrointestinal health and diarrhoeal disease<sup>13,14</sup> and mixed infections are common.<sup>14</sup> Additionally,  
80 the relative contribution of individual pathogens to disease burden varies across settlements, within  
81 a settlement over time, and between individuals. Interventions can also disrupt some transmission  
82 pathways more effectively than others.<sup>10</sup> The catch-all approach has traditionally been challenging  
83 due to the large number of possible enteropathogens, with a simpler solution to rely on bacterial  
84 indicator organisms to identify faecal contamination.<sup>15,16</sup> However, faecal indicators do not correlate  
85 well with pathogen abundance and distribution,<sup>17-20</sup> and reliance on indicators misses the complexities  
86 of enteropathogen diversity and pathogen-specific impacts of an intervention. Thus, the development  
87 of high-throughput molecular methods for enteropathogen screening of human, animal, and  
88 environmental samples would remove the need to rely solely on faecal indicators and can provide a  
89 comprehensive view of enteropathogen sources and diversity.

90 TaqMan quantitative PCR (qPCR) is a standard technique used across the human, animal, and  
91 environmental health fields to detect and quantify pathogens based on amplification of a pathogen-  
92 specific gene sequence.<sup>19,21-23</sup> This technique can be readily used to quantify pathogenic bacteria,  
93 viruses, protists, and helminths *in situ*, whereas alternative approaches such as selective cultivation,  
94 amplicon sequencing, and metagenomic sequencing are variably challenging to implement for non-  
95 bacterial targets. Moreover, given the ability to multiplex qPCR reactions and use 96-well and 384-  
96 well plates to process many samples at a time, this technique is relatively efficient, cheap, and high-  
97 throughput with respect to sample numbers. However, the price and labour time for screening many  
98 samples for several pathogens can become high, given each additional pathogen target adds to the  
99 cost of reagents, sample volume used, and preparation time. This can become prohibitive for  
100 enteropathogen detection across human, animal, and environmental samples, where the number and  
101 taxonomic diversity of enteropathogens contributing to the burden of disease may be high and is often  
102 unknown.<sup>24</sup>

103 The TaqMan Array Card (TAC), manufactured by Applied Biosystems, is a microfluidic card designed  
104 to automate several TaqMan qPCR assays per sample. Originally designed for gene expression  
105 experiments, TAC has been effectively repurposed for detection of large panels of pathogens,<sup>25</sup> with  
106 successful application to human faecal,<sup>26</sup> blood,<sup>27</sup> cerebrospinal fluid,<sup>28</sup> and nasopharyngeal<sup>29</sup> samples.  
107 Generally, the ability to efficiently detect large numbers of pathogens simultaneously is accompanied  
108 by a loss of sensitivity compared to standard qPCR,<sup>30,31</sup> though it is highly cost effective compared to  
109 standard qPCR for the breadth of targets that can be detected. Several large multi-centre studies have  
110 used TAC to study the aetiology of diarrhoeal disease.<sup>14,32</sup> However, TAC has rarely been applied to  
111 non-human samples, with only two studies to date using TAC to detect enteropathogens in food<sup>33</sup> and  
112 environmental<sup>18</sup> samples. The latter study showed that TAC can detect a wide range of pathogens in  
113 soil and water samples from informal settlements in Kisumu, Kenya.<sup>18</sup> However, the sensitivity,  
114 specificity, and quantitation accuracy of TAC has not yet been extensively evaluated in environmental  
115 samples in relation to the gold standard of qPCR. Thus, it is currently unclear whether the technique  
116 presents a valid alternative to standard qPCR to monitor multiple enteropathogens across different  
117 reservoirs.

118 In this study, we designed and evaluated a custom enteropathogen TAC that detects 46 different  
119 pathogen marker and faecal indicator genes. We first comprehensively tested the specificity,  
120 sensitivity, and accuracy of standard qPCR and TAC on a set of mock samples consisting of spiked  
121 enteropathogen genomic DNA in different sample matrices varying in PCR inhibition levels. We  
122 additionally tested both techniques on wastewater samples from Melbourne (Australia) and human  
123 stool, animal scat, environmental water, potable water, and soil samples from informal settlements  
124 in Suva, Fiji, where the diversity and prevalence of enteropathogens was not previously known.  
125 Through this approach, we demonstrate that TAC enables the reliable monitoring of multiple  
126 enteropathogens across environmental and host reservoirs.

127 **2. Materials and Methods**

128

129 **2.1. Spiked sample preparation and matrix testing**

130 Two sets of mock samples were prepared by spiking synthetic gene blocks (IDT, Australia) representing  
131 eight enteropathogen genes (**Table 1**; **Table S1**). Dilution of gene blocks for addition to each test  
132 matrix was conducted based on conversion of the measured nanograms of resuspended amplicon to  
133 total gene copy number using the formula:

134 number of copies (molecules) = 
$$\frac{X \text{ ng} * 6.0221 \times 10^{23} \text{ molecules/mole}}{(N * 660 \text{ g/mole}) * 1 \times 10^9 \text{ ng/g}}$$
 (1)

135 where X is the amount of measured amplicon (ng), N is the total length of dsDNA amplicon and 660  
136 g/mole represents the average mass of 1 bp dsDNA. Set 1 consisted of 10 nuclease-free water samples  
137 for comparison of method sensitivity and specificity. Three samples contained all eight targets at low  
138 (10 copies/µl), medium (100 copies/µl), or high (1000 copies/µl) concentration; six samples contained  
139 combinations of targets and concentrations; and one sample was a blank with no targets spiked.

140 Set 2 consisted of previously extracted Australian samples from different matrices to test the  
141 performance of the two methods under varying levels of PCR inhibition. The 36 samples tested  
142 included: nine wastewater samples with no gene blocks spiked; seven potable water samples spiked  
143 with 200 copies/µl of each target; and five different combinations of low, medium and high spiked  
144 targets in extracted DNA from each of four additional matrices (creek water, human stool, sediment  
145 and DNA extraction blank). All standard qPCR assays were performed on undiluted purified genomic  
146 DNA. As initial testing indicated that TAC reactions were inhibited for the wastewater and potable  
147 water matrices, these samples were assayed with TAC undiluted, diluted 1:10, and diluted 1:20 with  
148 nuclease-free water. Full details of the mock samples can be found in **Table S2**. All spiked samples  
149 were double-blinded and two separate laboratories delivered the results; one for TAC and another for  
150 standard qPCR.

151 **2.2. Fiji sample collection and processing**

152 Samples of child stool (n = 60), animal scats (n = 17), soil (n = 24), potable water (n = 10), and  
153 environmental water (n = 10) were collected from informal settlements in Suva, Fiji as part of the  
154 Revitalising Informal Settlements and their Environments (RISE) program (<https://www.rise-program.org>), a transdisciplinary research program and randomised controlled trial focused on  
155 improving environmental and human health in urban informal settlements of Fiji and Indonesia.<sup>34,35</sup>

157 Sixty child (< 5 year-old) stool samples were randomly selected for the current study from a total of  
158 287 samples collected from 12 informal settlements during the period September 27 to November 8  
159 2019. Samples were collected by the caregiver and stored at 4°C on frozen gel packs prior to transport  
160 to the laboratory and storage at -80°C within 24-48 hours.

161 Twenty water samples were collected in clean, source-water rinsed disposable bottles from the  
162 associated settlement. Potable water was run from local municipal water sources for 1 min prior to  
163 direct collection of 2 L of sample. Riverine, freshwater and stormwater (environmental water) samples  
164 were taken perpendicular from the bank and at an approximate depth of 0.15 m at each location. For  
165 each potable and environmental water sample, 1 L was filtered where possible through five 0.22 µM  
166 filters (Millipore). Where sediment prevented the passing of 1 L, a reduced volume was filtered until  
167 a total of five filters were collected. Filters were stored at -80°C within food-grade sealable bags.

168 Soil samples were collected using a sterile tongue depressor to transfer 2 cm<sup>3</sup> of material into food-  
169 grade sealable bags. Total animal stools were collected and stored in the same manner, with visual  
170 assessment of stool age to prevent collection of older “dry” samples. Material was placed at 4°C and  
171 transferred to the laboratory within two to four hours of collection. Animal scats and soil samples  
172 were homogenised (Stomacher 400 circulator, Seward) for 1 min at 250 × rpm and stored in 0.25 g  
173 aliquots in sterile cryo-storage tubes at -80°C.

174 For child stool, animal scats and soil samples, total genomic DNA was isolated from 0.25 g of material  
175 using the QIAGEN DNeasy PowerSoil Pro kit as per manufacturer’s instructions, and eluted in 50 µL of  
176 sterile molecular grade water. For water samples, the filters were crushed within the bags and  
177 transferred to the bead tubes with disposable spatulas for extraction with the QIAGEN DNeasy  
178 PowerMax Soil kit with the following modifications: after the addition of buffer C1, the samples were  
179 incubated at 65°C with shaking for 30 min at 200 rpm to lyse bacterial cells. The membranes were  
180 incubated for 10 min at room temperature in 1.5 ml of nuclease-free water prior to elution in this  
181 volume. Viral RNA is co-extracted with these kits. Extracted nucleic acid samples were frozen at -80°C  
182 prior to ambient transfer and refrigeration upon receipt in Melbourne, Australia. Samples were  
183 analysed by standard qPCR and TAC within one week without refreezing.

#### 184 **2.3. Standard TaqMan qPCR detection**

185 Standard TaqMan qPCR assays were undertaken using primers and probes for eight target pathogens  
186 (**Table 1**) under the PCR reaction and cycling conditions (40 cycles) described in US-EPA Method  
187 1696.<sup>36</sup> The PCR was conducted on a Biorad CFX96 thermocycler (Biorad, USA). Standard curves were  
188 prepared using the gene blocks (**Table S1**)<sup>37</sup> serially diluted 10-fold to achieve a five-point standard  
189 curve ranging from 10<sup>5</sup> to 10 copies/µL. Similarly, an internal amplification control gene block was  
190 diluted to a final concentration of 50 copies/µL with 100 copies added to each 25 µL reaction to  
191 indicate PCR inhibition.

192 Each 25 µL reaction contained 2 µL of either diluted standard gene block or sample genomic DNA.  
193 Reactions were conducted in triplicate for each sample and standard. Six replicates of no template  
194 controls were included on each run. The *Sketa22* assay described in Method 1696 was not performed  
195 as salmon testes DNA was not added prior to sample extraction. Quality control, data analysis and  
196 calculations were conducted as outlined in Method 1696<sup>36</sup> (using  
197 [https://www.epa.gov/sites/production/files/2019-04/methods-1696-1697-analysis-tool\\_march-2019.xlsm](https://www.epa.gov/sites/production/files/2019-04/methods-1696-1697-analysis-tool_march-2019.xlsm)), to ensure acceptance thresholds were met for R<sup>2</sup> (standards), amplification efficiency (E),  
198 no-template control (NTC), method blank, internal amplification control, and lower limit of  
199 quantification (LLOQ). Relative fluorescence units (RFU) analysis was conducted to ensure a peak had  
200 been generated for each target assay.  
201

#### 202 **2.4. TaqMan Array Card detection**

203 The custom TaqMan Array Card (TAC, Applied Biosystems) contained 48 singleplex assays (**Figure S1**;  
204 **Table S3**), including the eight primer and probe sets used in the standard qPCR assays and the  
205 manufacturer’s 18S rRNA control. Of the 47 custom assays, 40 have previously been validated on  
206 TAC<sup>26,38</sup> and the remaining seven were assays that have previously been published as individual  
207 TaqMan qPCR assays under similar conditions.<sup>14,39-41</sup> Cards were loaded with 100 µL of reaction mix  
208 per port, containing 60 µL of AgPath-ID One-Step RT-PCR master mix (Applied Biosystems; 50 µL buffer,  
209 4 µL enzyme mix and 6 µL nuclease-free water per port) mixed with 40 µL of sample nucleic acid.<sup>26</sup>  
210 Samples were diluted in nuclease-free water as necessary to allow a maximum of 1400 ng total nucleic  
211 acid per port, and 8 samples were tested per card. Loaded cards were centrifuged and sealed as per

212 manufacturer's instructions, and run on a QuantStudio 7 Flex instrument (Applied Biosystems) under  
213 the following cycling conditions: 45°C for 20 minutes, then 95°C for 10 minutes, followed by 45 cycles  
214 of 95°C for 15 seconds and 60°C for 1 minute.<sup>26</sup>

215 To calculate gene copies per microlitre, a standard curve was generated using synthetic plasmid  
216 controls (GeneWiz) as described in Kodani and Winchell *et al.* (2012).<sup>42</sup> Three plasmids were designed  
217 with primer and probe sequences included in inserts of approximately 1 kb each (15-20 targets per  
218 plasmid). If the primers or probe were degenerate, the sequence from the reference genome was  
219 used. All three plasmid insert sequences are in **Dataset S1**. The three plasmid controls were combined  
220 at equal concentrations and seven 10-fold serial dilutions were used to make the standard curve (7.2  
221 × 10<sup>6</sup> to 7.2 copies per microlitre). This positive control was run in triplicate with a no-template control  
222 on each card. Cycle threshold ( $C_q$ ) values were manually adjusted where necessary as below, with  $C_q$   
223 values exported to calculate a linear equation per target from the three replicates in R v3.6.2.<sup>43</sup> Note  
224 that a standard curve could not be generated for the manufacturer's 18S rRNA control. The lower limit  
225 of quantitation (LLOQ) was defined as the lowest dilution of the standard curve that was detectable  
226 in all three replicates. For quality control, one of the plasmids (not containing the rotavirus target)  
227 mixed with rotavirus A RNA was analysed with each new batch of master mix to test DNA polymerase  
228 and reverse transcriptase activity. A no-template control was included once every 10 cards to monitor  
229 reagent contamination.

230 TAC data were reviewed within the QuantStudio Real-Time PCR Software v1.3. Each multicomponent  
231 plot was manually checked for amplification, and  $C_q$  threshold values were checked and manually  
232 adjusted per target when the automatic threshold was inappropriate. Samples with very poor  
233 amplification curves were considered negative results, and wells flagged with both BADROX and NOISE  
234 or SPIKE, or another flag indicating a substantial issue with the well were omitted from analysis.  $C_q$   
235 values were exported and analysed in R v3.6.2<sup>43</sup> to calculate gene copies per microlitre of original  
236 nucleic acid extract using the standard curve for each target. For the purpose of this method  
237 comparison, all assays with a genuine amplification curve, regardless of  $C_q$  value, were considered  
238 positive.

## 239 **2.5. Sensitivity and specificity analyses**

240 Sensitivity and specificity for both methods were calculated using the spiked samples as follows:

$$241 \text{Sensitivity} = \frac{\text{true positives detected}}{\text{total spiked positives (i.e. true positives + false negatives)}} \quad (2)$$

$$242 \text{Specificity} = \frac{\text{true negatives detected}}{\text{total non-spiked negatives (i.e. true negatives + false positives)}} \quad (3)$$

243 To assess quantitation accuracy, the percentage of assays that quantified gene copies per microlitre  
244 within one  $\log_{10}$  of the spiked amount was calculated as follows:

$$245 |\log_{10}(\text{spiked copies}/\mu\text{l}) - \log_{10}(\text{measured copies}/\mu\text{l})| < 1 \quad (4)$$

246 For the second set of mock samples, specificity was not calculated because it was possible for spiked  
247 targets to already be present in the samples (false positives could not be determined). Additionally,  
248 samples with a background level of target detected by either method were excluded from the  
249 quantitation accuracy calculations.

## 250 **2.6. Statistical analysis**

251 All statistical analyses were performed in R v3.6.2.<sup>43</sup> Cohen's  $\kappa$  statistic was calculated with the  
252 kappa2() function from package irr v.0.84.1<sup>44</sup> to quantify agreement between standard qPCR and TAC

253 sensitivity. Wilcoxon's signed-rank test was applied with continuity correction using the `wilcox.test()`  
254 function.  $R^2$  values for concordance between measured qPCR and TAC gene copy numbers were  
255 calculated with the `lm()` function using  $\log_{10}$  transformed copy numbers with a pseudocount of 1 to  
256 accommodate values of 0. Graphics were created with `ggplot2 v3.3.2`.<sup>45</sup>

257 **3. Results**

258

259 **3.1. Assay sensitivity and specificity under ideal conditions**

260 General assay sensitivity and specificity was tested using synthetic gene blocks of eight pathogen  
261 markers spiked at different concentrations into nuclease-free water in ten different combinations (80  
262 individual assays; **Table S2 & S4**). Across all assays, the sensitivity of TAC was slightly lower (92%) than  
263 qPCR (100%). TAC performed well when detecting all eight targets at low concentration (10 copies/ $\mu$ l),  
264 but sometimes failed to detect targets at this concentration when others were present at high (1000  
265 copies/ $\mu$ l) concentration. Specificity was very high for both assays, with no false positives detected via  
266 TAC (100%) and one false positive detected by qPCR (94%). Both methods quantified spiked targets  
267 with variable accuracy in nuclease-free water, with TAC on average underestimating target abundance  
268 by 1.73-fold and qPCR by contrast overestimated target abundance by 2.61-fold (**Figure 1; Table S4**).  
269 Overall, 98.75% of all qPCR results within one log of the spiked concentration, compared to 91.25%  
270 from TAC (**Table 2**). Most (5/7) of these differences from the TAC results were instances of low-copy  
271 targets that were not detected by TAC. It should be noted that the layout of TACs prevent the  
272 generation of a standard curve for each run and  $C_q$  thresholds are applied independently to each card.  
273 However, minimal run-to-run variation was observed amongst the plasmid controls, with controls  
274 providing very similar  $C_q$  values across cards; the lowest dilution, 7.2 gene copies per microlitre, was  
275 the most variable and for some targets was not consistently detected. Altogether, these findings  
276 suggest that TAC and qPCR perform comparably in inhibitor-free sample matrices.

277 **3.2. Assay performance in inhibited sample matrices**

278 The second set of test samples was used to determine the performance of each technique on samples  
279 with varying levels of PCR inhibition (**Table S2 & S4**). For both methods, there was a reduction in  
280 sensitivity (77.3% TAC and 89.2% qPCR of spiked targets detected) and quantitation accuracy (66.7%  
281 TAC and 69.3% qPCR assays within one log of the spiked concentration) across all sample matrices  
282 (**Table 2**). This decrease in performance compared to samples spiked in nuclease-free water (**Figure**  
283 **1**) suggests both methods, especially TAC, are affected by PCR inhibitors. There was nevertheless much  
284 variability in the relative performance of the two methods across different sample matrices and  
285 pathogen targets. For example, while TAC underperformed relative to qPCR in spiked fluorinated  
286 potable water samples, the converse was true for sediment samples. Likewise, while TAC detected  
287 *Cryptosporidium* with higher accuracy, qPCR was more sensitive and accurate for detecting  
288 *Campylobacter* (**Table 2**). TAC also detected a range of indicators and pathogens present in Melbourne  
289 sewage and stormwater samples (**Figure 3**). TAC was more inhibited by this sample matrix than qPCR  
290 and detected no targets (including universal 16S rRNA) in five of the eight samples. However, diluting  
291 samples (1:10 and 1:20) greatly improved detection for all samples, resulting in 216-fold and 273-fold  
292 increases in the number of targets detected respectively (**Table S5**). These results suggest that TAC is  
293 generally, though not consistently, less sensitive and accurate than qPCR for sample matrices with  
294 high inhibitor content. In common with previous findings,<sup>18</sup> however, sample dilution greatly reduced  
295 inhibition without compromising detection for moderately to highly abundant targets.

296 **3.3. Performance comparison with faecal and environmental samples from urban informal**  
297 **settlements in Fiji**

298 A set of 121 samples from informal settlements in Fiji consisting of 60 child stool, 17 animal scats  
299 (predicted to be primarily from dogs and ducks), 20 water (10 environmental, 10 potable) samples,  
300 and 24 soil samples were analysed with TAC and standard qPCR. The nature and distribution of  
301 enteropathogen contamination in this environment is relatively unknown, and this sampling effort  
302 represents an initial insight into the baseline conditions of these settlements prior to the water and  
303 sanitation intervention to be trialled by the RISE program.<sup>34,35</sup> The full dataset containing measured  
304 gene copies per microlitre is provided in **Table S6**. For the eight pathogen targets assayed by both  
305 methods, the presence/absence concordance rate was high, with 89% of all assays in agreement  
306 between both methods (Cohen's  $\kappa = 0.619$ ) (**Figure 2a**). Of the remaining discordant 11%, 7%  
307 represented a detection by qPCR that was not observed with TAC, and 4% a TAC detection missed by  
308 qPCR; this indicates that the greater overall sensitivity of qPCR does not preclude the ability for TAC  
309 to detect pathogens when qPCR does not. Only one sample (a child stool) was indicated to be  
310 significantly inhibited by the qPCR *Bacteroides* internal amplification control; despite this, both  
311 methods detected the *Bacteroides* faecal indicator.

312 For assays where both methods detected the target, quantitation is quite consistent with  $R^2 = 0.815$   
313 (**Figure 2b**). The distribution of measured quantities for targets detected by only one method is similar  
314 on both axes, indicating that both qPCR and TAC can similarly detect targets that are missed by the  
315 other method. The target quantities measured by the two methods were significantly different ( $p =$   
316 0.00006, Wilcoxon signed rank), which was driven by instances where a target at low concentration  
317 was detected by one method and not the other (**Figure 2b**). When considering the concordance  
318 between the techniques when a quantity was measured by both, differences in quantities were not  
319 statistically significant ( $p = 0.206$ , Wilcoxon signed rank).

320 The pathogens detected in each sample type are shown in **Figure 2a**. All targets were found in at least  
321 four samples. *Giardia* and enteropathogenic *E. coli* (EPEC; *eae* gene) were the most common of the  
322 eight targets, whereas *Salmonella* and *Cryptosporidium* were found infrequently. Reflecting the  
323 concordance rate, detections of each target in each sample type were similar. The generic faecal  
324 indicator *Bacteroides* was detected more often by TAC than by qPCR, especially in child stool samples.  
325 The major discrepancy in results was the reported detection of *Giardia* in several soil samples by qPCR  
326 (quantified at 10-100 copies per microlitre of original sample), which were not detected by TAC. It is  
327 possible that these hits are true positives that were not detected by TAC due to a combination of low  
328 *Giardia* levels, sample dilution, and challenging detection in a soil matrix. However, it is also possible  
329 that false positive detection underlies these issues given *Giardia* qPCRs accounted for the only false  
330 positive detected in the spiking study (**Figure 1**) and thus further work is required to discriminate this.  
331 Negative extraction controls were free of amplification, with the exception of a low concentration of  
332 *Giardia* in the animal scat control (detected by both methods) and in the soil control (detected only  
333 by qPCR).

### 334 **3.4. Detection of other pathogens by TAC**

335 In addition to the eight targets assayed via both methods, the custom TAC was designed to detect a  
336 range of other viral, bacterial, protist, and helminth enteropathogen targets (**Figure S1; Table S3**). Of  
337 the 48 targets on the card, 44 were detected at least once (39 pathogen targets, 3 faecal indicators, 2  
338 controls); astrovirus, *S. enterica* serovar Typhi (Typhoid fever), *Necator americanus* (hookworm), and  
339 *Cystoisospora belli* (isosporiasis) were not detected in any sample. Overall, most samples contained a  
340 wide range of enteropathogens (**Figure 3**), with the exception of potable water which contained only  
341 very low concentrations of human faecal indicators. Environmental water and animal scat samples  
342 were the most rich in enteropathogens, with most samples containing more than eight pathogens and

343 ten targets (**Figure 3c**). Somewhat fewer pathogens were detected in child stool and soil (av. 2.8 and  
344 2.2 pathogens per sample respectively, excluding faecal indicators).

345 The most prevalent enteropathogens across host and environmental reservoirs were  
346 enteroaggregative *E. coli* (EAEC) and EPEC. All three target genes were commonly detected for EAEC  
347 (*aaiC*, *aatA*, *aggR*), whereas the *eae* gene was detected more frequently than *bfpA* for EPEC (**Figure**  
348 **2a**). Both markers of *Shigella flexneri* clade 6 (O-antigen, type 3 restriction enzyme) were also present  
349 in 18 samples.<sup>14</sup> *Giardia* and *Blastocystis* were common protists, with *Giardia* present at highest  
350 concentrations in human stool (**Figure 2b**). Amongst helminths, the large roundworm *Ascaris* was  
351 most common in human faecal samples, which is concordant with findings that this genus infects  
352 approximately a sixth of the world's population.<sup>46</sup> In contrast, *Ancylostoma* and *Trichuris* were  
353 predominant in animal faeces. Few helminths were detected in soil, but those that were present had  
354 moderately high abundance (approximately 120-140 copies per ng of DNA). Viruses were less  
355 prevalent overall; rotavirus and adenovirus F were most commonly detected, primarily in  
356 environmental water, whereas norovirus GII was abundant in one stool sample (**Table S6**). Some other  
357 targets, notably *Campylobacter* pan, *Entamoeba* pan, *Aeromonas*, and *Plesiomonas shigelloides*, were  
358 abundant in environmental waters and other samples. However, they were infrequently found in child  
359 stool.

360 TAC also detected a range of faecal indicators and other marker genes. The universal bacterial marker  
361 16S rRNA was detected in most samples, absent in only one environmental water and all potable water  
362 samples. Providing an estimate of total bacterial load, 16S rRNA quantities were highest in the human  
363 faecal samples and lowest in environmental water. Aside from the universal 16S rRNA assay, the most  
364 common target detected overall was human-associated *Lachnospiraceae*, a faecal marker detected in  
365 the majority of child stool and environmental water samples. This suggests that *Lachnospiraceae* is a  
366 useful faecal indicator in this population, in agreement with previous studies.<sup>39,47</sup> CrAssphage, an  
367 abundant bacteriophage of human *Bacteroides* and a proposed faecal indicator,<sup>41,48</sup> was the least  
368 common faecal indicator detected in individual stool samples, but was present in most of the  
369 environmental water samples. These reportedly human-specific faecal indicators were also detected  
370 in the animal scats, though to a lesser extent than in child stool (**Figure 2**).

371

372 **4. Discussion**

373 Until now, the TaqMan Array Card has been validated for pathogen detection in human clinical  
374 samples,<sup>26,30,31</sup> and has, to the best of our knowledge, been used in only two studies to date to detect  
375 enteropathogens in non-human samples.<sup>18,33</sup> In the current study, to evaluate the performance of TAC  
376 compared to standard qPCR on environmental samples, we compared enteropathogen assays with  
377 both techniques using spiked samples of known concentration, wastewater samples from Melbourne  
378 (Australia), and a range of sample types collected from urban informal settlements in Fiji. We found  
379 that the performance of TAC in environmental samples was comparable to standard qPCR with respect  
380 to specificity, sensitivity, and quantitation accuracy in clean sample matrices. Nevertheless, TAC was  
381 somewhat less sensitive than standard qPCR in detecting spiked targets in matrices with variable  
382 inhibition. Both assays varied in quantitation accuracy depending on sample matrix and pathogen  
383 target, with TAC underestimating abundance by 1.13-fold and qPCR overestimating abundance by  
384 1.48-fold on average across the entire dataset of spiked samples. The capacity of TAC to efficiently  
385 detect multiple enteropathogen targets potentially counterbalances these tradeoffs in sensitivity and  
386 accuracy, given the benefits of monitoring a large array of enteropathogens in samples from heavily  
387 contaminated environments. In addition, we show that TAC can effectively quantify enteric pathogens  
388 across a range of environmental, human, and animal reservoirs, thereby providing a unified method  
389 to monitor pathogen transmission pathways and evaluate public health interventions.

390 It can be expected that TAC would detect fewer targets per sample than qPCR. The smaller reaction  
391 volume for TAC (approximately 1 µl compared to 20 µl standard qPCRs) means there is a reduced  
392 chance that a reaction well contains a copy of a low-concentration target. Reduction in sensitivity has  
393 been observed in previous comparisons between standard qPCR and TAC. For example, Kodani *et al.*  
394 (2011) compared standard qPCR and TAC performance on respiratory specimens, and observed a  
395 general 10-fold reduction in sensitivity with TAC; some assays had a greater drop in sensitivity while  
396 others were as sensitive as standard qPCR.<sup>49</sup> Subsequent studies have also reported significant  
397 reductions in sensitivity for TAC.<sup>30,31</sup> Importantly, some of these previous standard qPCR/TAC  
398 comparisons have evaluated the performance of TAC relative to the standard qPCR, rather than a side-  
399 by-side comparison.<sup>31,49</sup> This means that the sensitivity and accuracy of standard qPCR is assumed to  
400 be 100% (as the gold standard), whereas that for TAC is reported as the percentage of assays that  
401 agree with standard qPCR results. This may be a reasonable approach for diagnostics in clinical  
402 samples, though our tests with spiked environmental sample matrices indicated that the performance  
403 of standard qPCR is not optimal; this method overestimated target abundance overall, falsely detected  
404 *Giardia* in at least one sample, and performed suboptimally in some matrices (e.g. fluorinated water,  
405 sediment). By evaluating both methods independently, we provide a clearer view of how they each  
406 perform in challenging sample types and demonstrate that they remain comparable.

407 TAC was generally more susceptible to inhibition by PCR inhibitors than standard qPCR in both the  
408 spiked samples and Melbourne wastewater samples. However, sample dilution by 1:10 and 1:20 was  
409 sufficient to alleviate this and yield strong amplification curves for a wide range of indicators and  
410 pathogens. By contrast, we detected minimal inhibition in the stool, scat, soil, and water samples  
411 extracted from the Fiji informal settlements with DNeasy kits, as verified by a standard qPCR internal  
412 control. We recommend that environmental studies test for PCR inhibition prior to application of TAC  
413 to enable appropriate changes in sample preparation, such as dilution, or re-extraction of samples  
414 with optimised methods. For example, studies led by Baker<sup>18</sup> and Tsai<sup>33</sup> initially screened for  
415 inhibition with the QuantiFast pathogen kit and used 1:10 dilution to resolve it if present. Alternatively,  
416 it is possible to introduce internal amplification controls on TAC. However, we elected not to include  
417 one for this study due to the concern that the addition of control DNA would impact the detection of

418 other targets, as enteropathogens in real environmental samples were likely to be at low  
419 concentrations and the reaction volume is minimal. Instead, we included a universal 16S rRNA target  
420 in addition to the manufacturer's 18S rRNA control; failure of these targets to amplify indicates either  
421 substantial inhibition or minimal sample biomass, which can be discriminated by quantifying DNA  
422 through spectroscopic methods (e.g. Nanodrop, Qubit).

423 As both the TAC and qPCR approaches evaluated here both use the same fundamental method of  
424 TaqMan qPCR to specifically target pathogen genes of interest, both techniques are excellent options  
425 for environmental enteropathogen monitoring. As summarised in **Table 3**, they have different  
426 strengths and limitations depending on purpose of their applications. Standard qPCR offers greater  
427 sensitivity and the ease of running replicates to improve confidence in positive results and quantities,  
428 while TAC is capable of detecting up to 47 custom targets across 8 samples in a minimally laborious  
429 way, eliminating pipetting error, and greatly reducing the potential for assay contamination. It is,  
430 however, important to consider the limitations of both methods. An overarching caveat is that TAC is  
431 designed to provide the best overall result and will not be optimal for individual pathogens;  
432 optimisation of assays is more easily done for standard qPCR. Quantitation is ideally performed using  
433 a standard curve included on each run, with quality control measures associated with this.<sup>36</sup> As a TAC  
434 standard curve can only occasionally be produced (requiring one whole card per replicate), the  
435 quantitation is less stringent and standard curve-related quality control measures not applicable. We  
436 demonstrated with spiked gene blocks that accurate quantitation with TAC is achievable, but the  
437 assays that target ribosomal RNA will amplify an unknown number of gene copies per organism given  
438 rRNA copy number variation.<sup>50</sup> Moreover, as the protocol involves a universal reverse transcription  
439 step to detect RNA viruses, both gene and mRNA copies of all targets will be amplified. This makes it  
440 challenging to accurately estimate the number of organisms per gram or millilitre of original sample,  
441 but potentially boosts sensitivity. Some of the gene targets for bacterial pathogens assayed here are  
442 encoded on plasmids, which can be of high copy number and hence further compromise quantitation.  
443 Finally, an important consideration when interpreting qPCR-based pathogen detection is that it does  
444 not indicate organism viability. Persistence of DNA from non-viable pathogens in the environment is  
445 likely to vary by organism, and presence of DNA in stool samples without active clinical infection can  
446 also complicate interpretation of results.<sup>51</sup>

447 In the context of assessing water and sanitation interventions in low-income contexts, however, a  
448 consistent method measuring relative change over time without the need to calculate organism  
449 numbers or identify aetiology is appropriate. We also included as many enteropathogen targets as  
450 possible, with no replicates. Customising cards to include fewer targets in duplicate, for example, may  
451 improve TAC sensitivity further. Liu *et al.* (2013) reported that almost half of low-concentration targets  
452 spiked into stool were detected in only one of two replicates,<sup>26</sup> which is in agreement with the  
453 variability in the lowest dilution of our TAC standard curve. The feasibility of reducing the number of  
454 targets to introduce replicates is a trade-off dependent on the research project and the samples to be  
455 screened, and it may be beneficial to have some assays also available via standard qPCR to confirm  
456 ambiguous results or the presence of inhibition. Despite these limitations, we have shown TAC to  
457 perform comparably and have several advantages over standard qPCR that encompass the ability to  
458 scale up the number of targets with minimal impact on the amount of sample required, cost per  
459 sample, risk of assay contamination and error, and overall ease-of-use.

460 We demonstrated that there is a diverse range of enteropathogens present in urban informal  
461 settlements in Suva, Fiji, highlighting the utility of TAC in this setting. There was a high burden of  
462 bacterial pathogens in human stools, particularly EAEC and EPEC, as well as wide range of bacterial,  
463 protist, and helminth pathogens in environmental waters and animal scats. The inclusion and

464 detection of soil-transmitted helminths in both human and environmental samples on TAC is a  
465 particularly important advance, as the standard methods for detection of such pathogens in stool  
466 involve conventional microscopy (labour-intensive, subjective, and high-risk for the operator) or  
467 serology (only viable for human-derived samples).<sup>52</sup> Despite their transmission pathway, soil-  
468 transmitted helminths were more common in human and animal faeces than in soil samples from Fiji.  
469 The soil samples taken may not have been representative of helminth-contaminated areas, or  
470 alternatively detection may have been influenced by the integrity of helminth eggs impeding DNA  
471 extraction or the difficulties of detecting pathogens with low abundance in soil (demonstrated by our  
472 mock samples) combined with the small volume of soil extracted. Regarding human faecal indicators,  
473 *Lachnospiraceae* seems most suitable for this population as it was detected in the majority of faecal  
474 samples. As the assay used has been validated as highly human-specific,<sup>39</sup> its presence in animal scats  
475 suggests a close association between animals and people in these settings, or possibly  
476 misidentification of scat origin. While *Bacteroides* and CrAssphage were less frequently detected in  
477 individual child stools, all three indicators were common in environmental waters. Through RISE, we  
478 are conducting further studies to understand the distribution and transmission of enteropathogens in  
479 the Fiji sites, as well as the trial settlements in Makassar, Indonesia.<sup>35</sup>

#### 480 Conclusion

481 Techniques that can adequately monitor a range of enteropathogens in humans, animals and the  
482 environment are required to assess water and sanitation improvements that aim to interrupt diverse  
483 transmission pathways. The use of qPCR in the form of individual assays or via the TaqMan Array Card  
484 enables direct detection of several enteropathogens in a range of sample types, bypassing reliance on  
485 faecal indicator organisms. Our study is the first to our knowledge to evaluate the performance of a  
486 custom TAC compared to standard qPCRs on human, animal, and environmental samples. We have  
487 demonstrated that, in these challenging sample matrices, TAC is comparable to standard qPCR and is  
488 a cost-effective, scalable, accurate, and easy to use alternative for multiple pathogens. Better  
489 understanding of the distribution, transmission, and impacts of a broad range of enteropathogens  
490 across environmental, human, and animal reservoirs is essential for improvements to public health  
491 towards SDGs 3 and 6. Among various potential applications, this will be critical for informing and  
492 evaluating future water and sanitation interventions in urban informal settlements, where the nature  
493 and extent of enteropathogen contamination is poorly characterised and diverse. More broadly, this  
494 technology enables unified approaches for surveying enteropathogens in populations and  
495 environments, as well as resolving and interrupting their transmission pathways.

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656

657 **Tables**

658 **Table 1.** TaqMan qPCR assays used for detection by standard qPCR and custom TaqMan Array Cards.

Organism	Targeted gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	TaqMan probe (standalone qPCR) <sup>a</sup>
<i>Campylobacter jejuni / coli</i>	<i>cadF</i>	CTGCTAAACCATA GAAATAAAATTCT CAC	CTTGAAAGGTAATT TAGATATGGATAA TCG	5'HEX- CATTGACGATTTGG CTTGA-3'MGB
<i>Salmonella enterica</i>	<i>invA</i>	TCGGGCAATTGTT ATTGG	GATAAACTGGACC ACGGTGACA	5'FAM- AAGACAACAAACCCAC CGC-3'MGB
Shiga toxin-producing <i>Escherichia coli</i> (STEC)	<i>stx1</i>	ACTTCTGACTGCA AAGACGTATG	ACAAATTATCCCCT GWGCCACTATC	5'Texas Red- CTCTGCAATAGGTACTCC A-3'MGB
STEC	<i>stx2</i>	CCACATCGGTGTCT GTTATTAACC	GGTCAAAACGCGC CTGATAG	5'FAM- TTGCTGTGGATATACGA GG-3'MGB
Enteropathogenic <i>E. coli</i> (EPEC)	<i>eae</i>	CATTGATCAGGATT TTTCTGGTGATA	CTCATGCGGAAT AGCCGTTA	5'FAM- ATACTGGCGAGACTATT CAA-3'MGB
<i>Cryptosporidium</i>	18S rRNA	GGGTTGTATTATT AGATAAAGAACCA	AGGCCAATACCC ACCGTCT	5'FAM- TGACATATCATTCAAGTT TCTGAC-3'MGB
<i>Giardia</i>	18S rRNA	GACGGCTCAGGAC AACGGTT	TTGCCAGCGGTGT CCG	5'HEX- CCCGCGGCGGTCCCTGC TAG-3'MGB
<i>Bacteroides</i>	16S rRNA	ATCATGAGTTCACA TGTCCG	CTTCCTCTCAGAAC CCCTATCC	5'FAM- CTAATGGAACGCATCCC- 3'MGB
Internal amplification control <sup>b</sup>	16S rRNA	ATCATGAGTTCACA TGTCCG	CTTCCTCTCAGAAC CCCTATCC	5'VIC- AACACGCCGTTGCTACA- 3'MGB

659 <sup>a</sup> TaqMan Array Card probes were identical with the exception of the fluorophore (all TAC probes  
660 were 5'FAM 3'MGB).

661 <sup>b</sup> Internal amplification control targets *Bacteroides* and was applied to standalone qPCR assays only.

662 **Table 2.** Performance of TAC and qPCR on spiked samples in sample matrices varying in levels of PCR  
663 inhibitors. Results are shown by sample matrix and by target. Sensitivity is defined as the percentage  
664 of spiked targets that were detected. Accuracy is measured as percentage of assays within one  $\log_{10}$   
665 of the spiked concentration; assays where background levels of pathogen were detected by qPCR or  
666 TAC are excluded from these calculations.

667

Sample matrix	Sensitivity (TAC, %)	Sensitivity (qPCR, %)	Accuracy (TAC, %)	Accuracy (qPCR, %)
Nuclease-free water	92.2	100	91.3	98.8
Creek water	93.3	96.7	80.0	80.0
Sediment	60.0	56.7	42.5	30.0
Human stool	83.3	96.7	80.0	90.0
Fluorinated potable water	71.4	94.6	62.5	73.2
Extraction blank	83.3	96.7	80.0	82.5
Target				
<i>Bacteroides</i>	85.2	85.2	81.5	85.2
<i>Campylobacter jejuni / coli</i>	77.4	87.1	48.6	78.4
<i>Cryptosporidium</i>	77.4	90.3	70.3	54.1
EPEC ( <i>eae</i> )	74.2	90.3	73.0	78.4
<i>Giardia</i>	90.3	96.8	81.5	81.5
<i>Salmonella</i>	83.9	96.8	78.1	68.8
STEC ( <i>stx1</i> )	85.2	96.3	87.5	90.6
STEC ( <i>stx2</i> )	77.4	93.5	78.4	91.9

668

669 **Table 3.** Comparison of TAC vs qPCR for monitoring multiple pathogens.

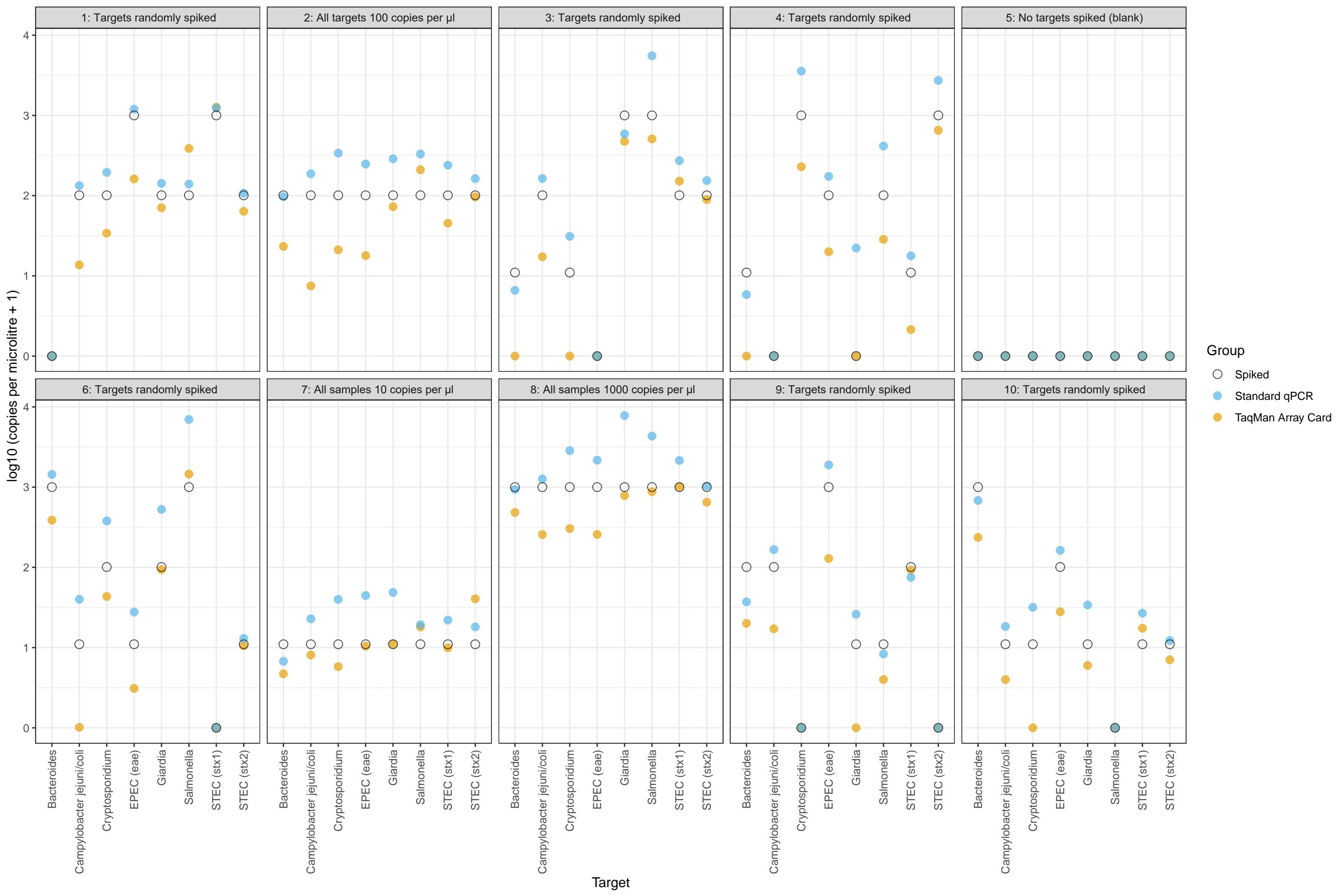
Method	Quantitative PCR (qPCR) and reverse transcriptase qPCR (RT-qPCR)	TaqMan Array Cards (TAC)
Target range	<b>Narrow target range:</b> Individually detects any target of interest with appropriately optimised primers/probes. However, adding additional targets requires extra sample volume, labour, cost, and plastic waste. Assays can be multiplexed (detection of multiple targets in one reaction) with careful optimisation.	<b>Broad target range:</b> Simultaneously detects up to 47 targets and 1 internal control across 8 samples. Assays require careful card design and manufacture with appropriate lead-time. Optimisation may be required for target quantitation under universal conditions on the card.
Sensitivity / accuracy	<b>High sensitivity and accuracy:</b> Theoretical detection limit is approximately three gene copies per reaction. Pathogen quantitation possible with appropriate reference standards. PCR inhibition possible, but can be readily monitored with controls.	<b>High sensitivity / medium accuracy:</b> Sensitivity high but often lower than qPCR given smaller reaction volume and universal reaction conditions. Pathogen quantitation possible with reference standards, but generally requires comparisons between cards. Quantitation also challenging due to co-detection of DNA and RNA due to universal reverse transcriptase step to detect RNA viruses. Greater potential for PCR inhibition.
Specificity	<b>High specificity:</b> Well-designed TaqMan primer and probe sequences are very specific.	<b>High specificity:</b> Same TaqMan technology as standard qPCR.
Scalability	<b>Moderate scalability:</b> Extensive manual handling with large numbers of samples and/or pathogens. Large sample numbers require high labour time or robotics. Increased sample numbers require greater sample volume and produce more waste. High potential for pipetting errors.	<b>High scalability:</b> Simple and moderately fast (~3 hours) to prepare and run from extracted nucleic acids. Labour time is minimal given the few manual handling tasks required, though increases per card (eight samples). Low potential for pipetting errors.
Cost	<b>Low cost per sample:</b> Low reagent cost per sample (approximately USD \$2.10 for one pathogen without replicates). Small cost increase with more samples, but large increase with more targets (double the labour and reagents cost for two targets).	<b>Low cost per pathogen:</b> Moderate reagent cost per sample (approx. USD \$60). However, highly cost effective for monitoring multiple targets per sample (approx. USD \$1.28 per sample per target without replicates).
Resources	<b>Moderate resources:</b> Requires real-time thermal cycler. Training for molecular biology, equipment use, and software required.	<b>Moderate resources:</b> Requires real-time thermal cycler with array card block. Training needed for molecular biology, equipment, and software.

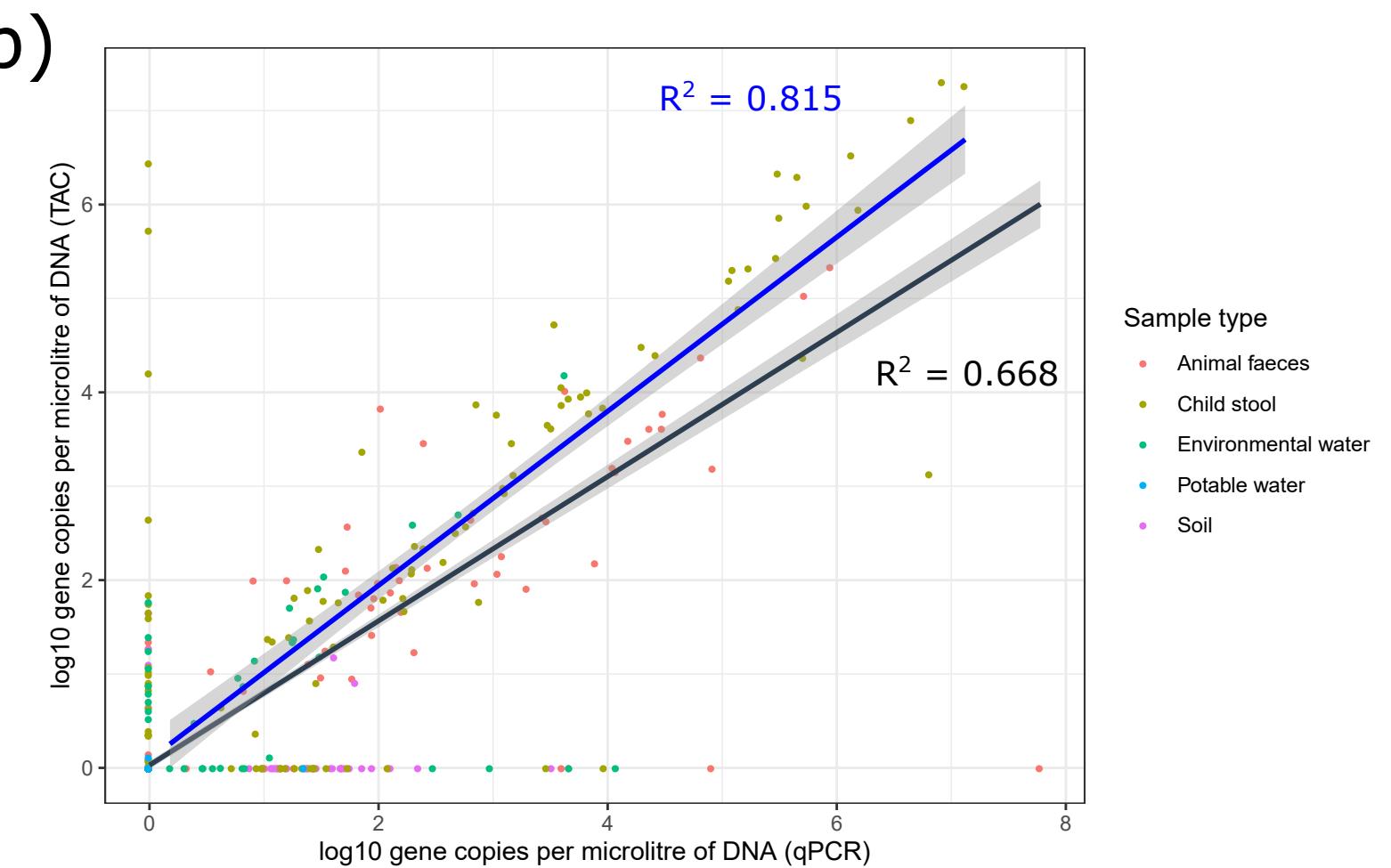
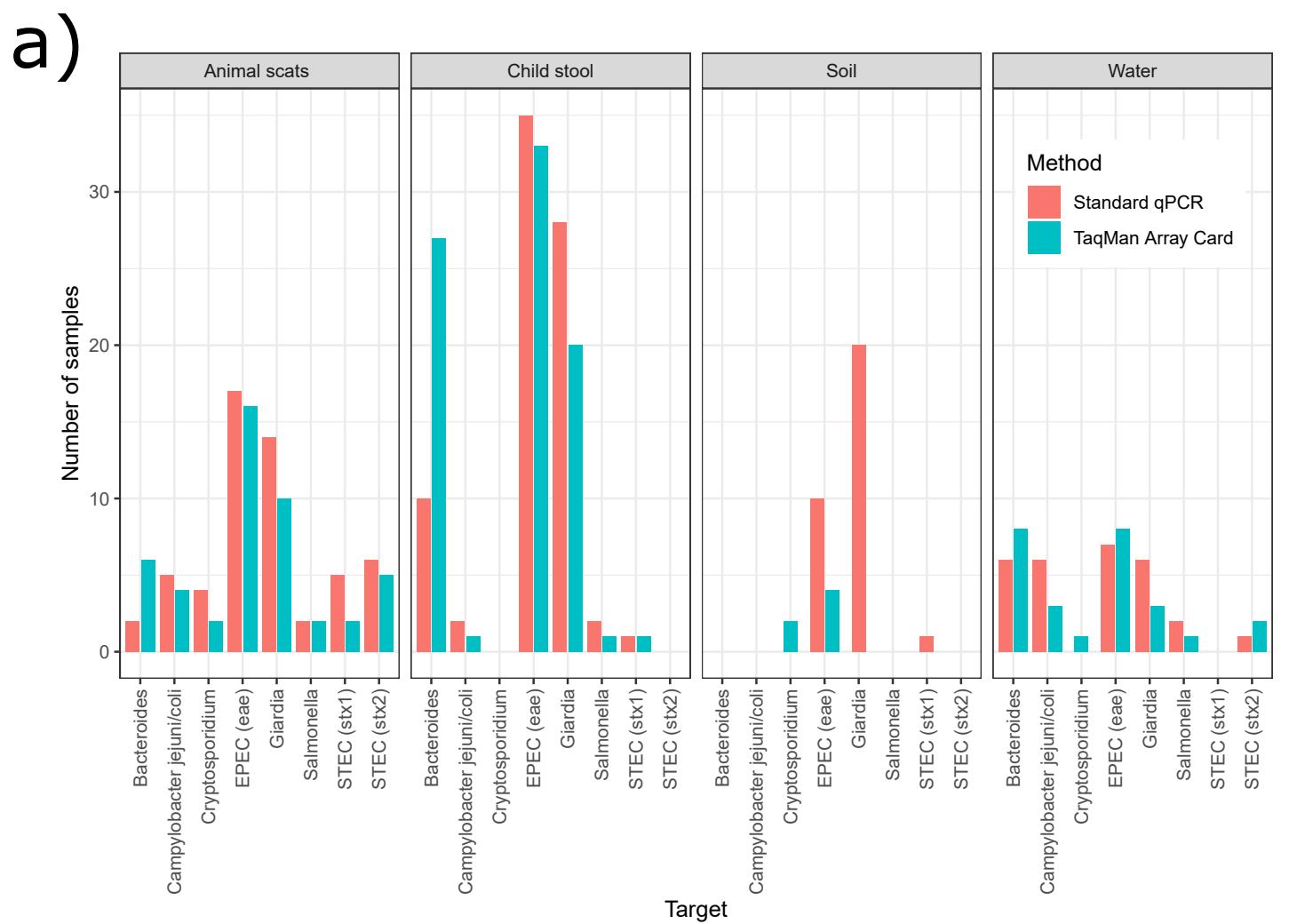
671 **Figure legends**

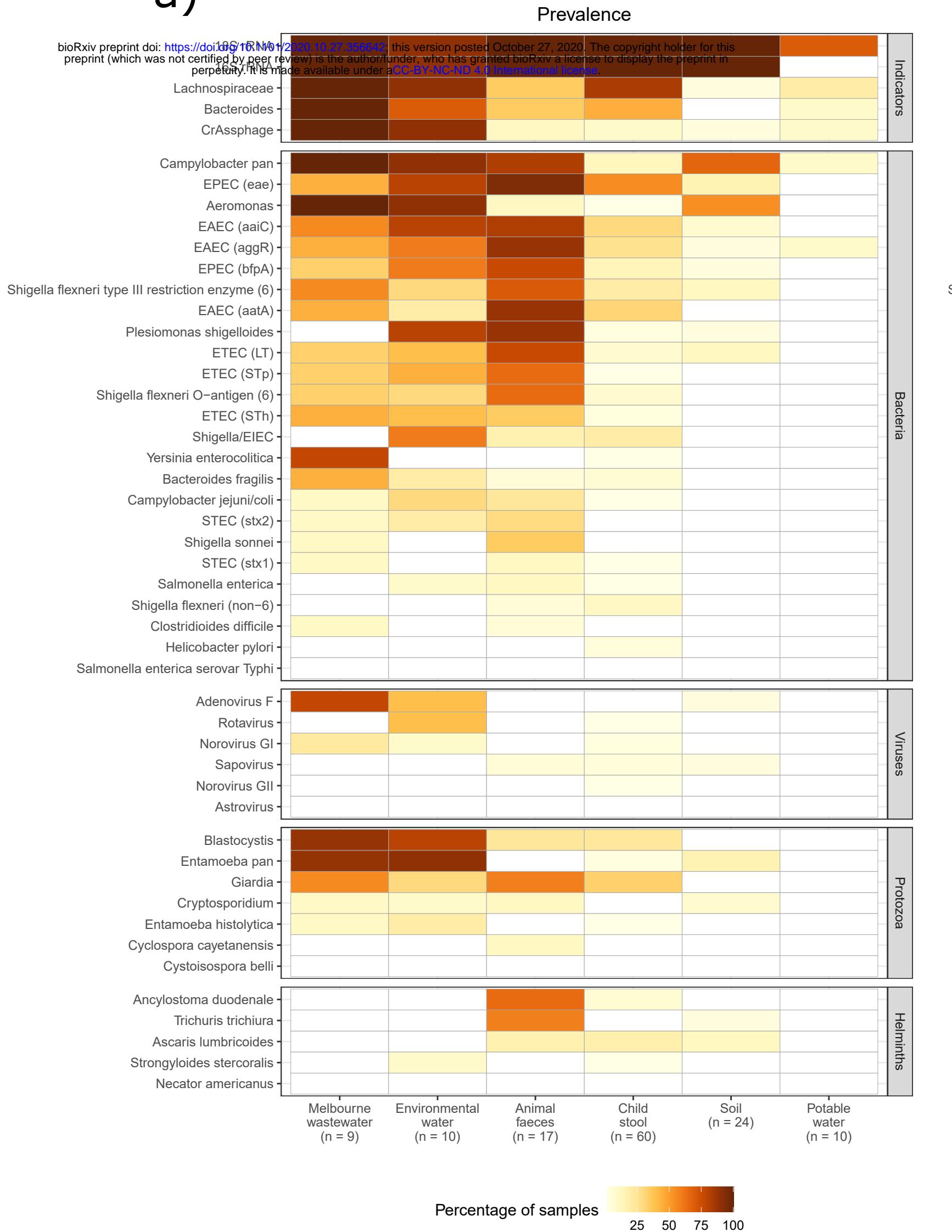
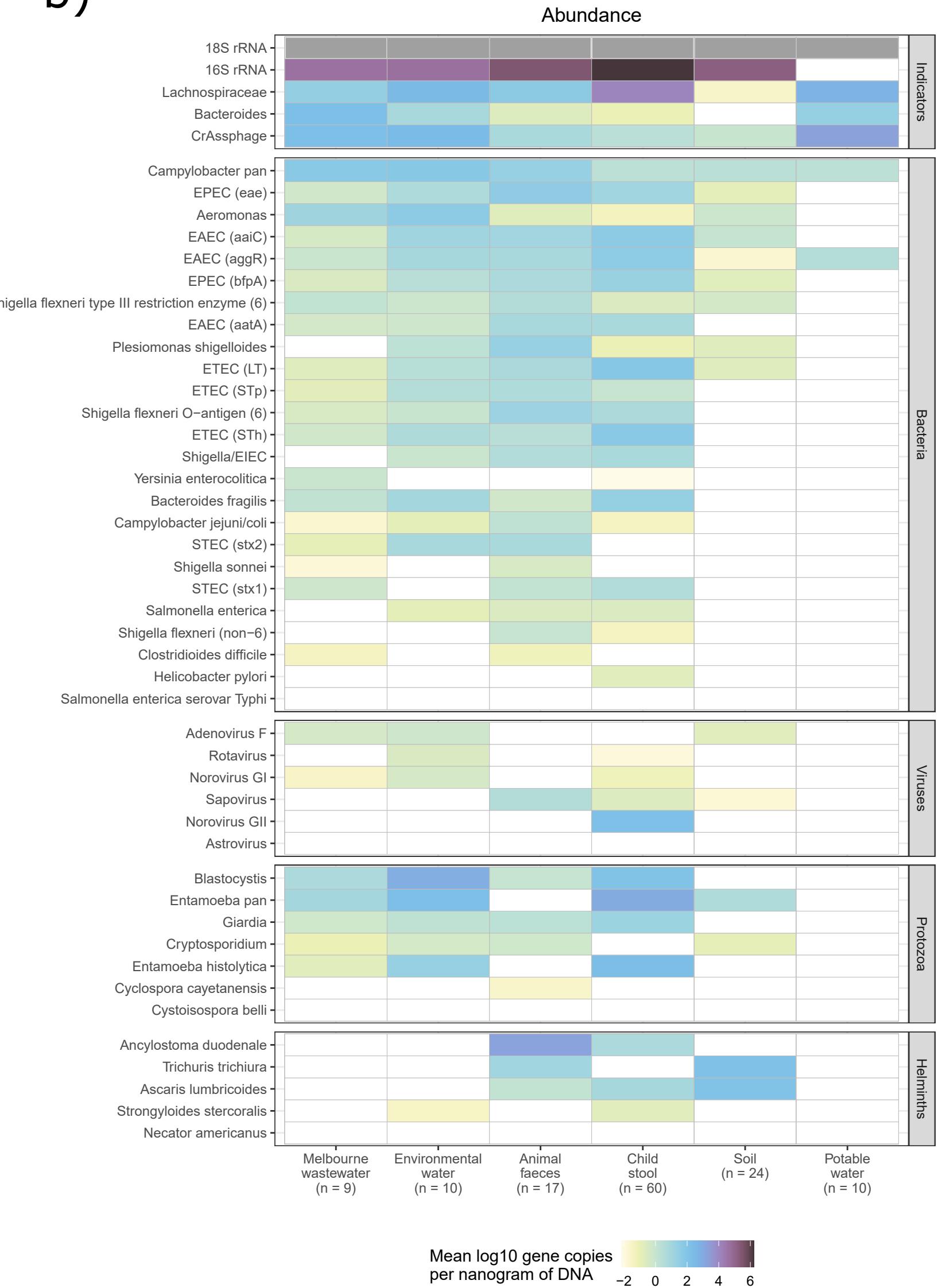
672 **Figure 1.** Quantitation of spiked genetic material in nuclease-free water by TAC and standard qPCR. Ten  
673 different combinations of spiked material were tested in a randomised double-blinded manner. Targets  
674 were either: spiked randomly in different combinations (samples 1, 3, 4, 6, 9, 10); spiked at consistent  
675 concentrations of 10 (sample 7), 100 (sample 2), or 1000 (sample 8) copies per microlitre; or not spiked  
676 at all (sample 5; a blank control). For each target, the quantity of material spiked (white circle), the copies  
677 detected by standard qPCR (blue circle), and the copies detected by TAC (yellow circle) are shown.

678 **Figure 2. Concordance between standard qPCR and TAC in detecting pathogens in animal scats, child  
679 stool, soil, and water collected from informal settlements of Suva, Fiji.** Agreement between the methods  
680 with respect to **a**) the number of positive detections of targets and **b**) the measured target quantity in  
681  $\log_{10}$  gene copies per microlitre of extracted DNA (with a pseudocount of 1 added before  $\log_{10}$   
682 transformation). The regression lines with associated 95% confidence intervals are shown for the subset  
683 of data where a target was quantified by both methods (blue,  $R^2 = 0.815$ ). Across all data points,  $R^2 =$   
684 0.668 (black).

685 **Figure 3. Pathogen and indicator targets detected via TaqMan Array Card in Melbourne wastewater  
686 samples and animal scats, child stool, soil, and water collected from informal settlements of Suva, Fiji.**  
687 Heatmaps represent the **a**) prevalence (percentage of positive samples) and **b**) abundance (mean value  
688 of  $\log_{10}$  gene copies per nanogram of DNA across positive samples) of each target by sample type. White  
689 represents a zero value, and 18S rRNA quantitation was unavailable. The number of pathogens or  
690 indicators detected per sample is represented by **c**) histograms, also by sample type. This excludes 16S  
691 rRNA and 18S rRNA and counts pathogens with multiple gene targets once.





**a)****b)****c)**