Impact of DNA polymerase choice on assessment of bacterial communities by a *Legionella* genus-specific next-generation sequencing approach

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ABSTRACT

The library preparation step is a major source of bias in NGS-based studies. Several PCR-related factors might negatively influence the application of NGS tools in environmental studies and diagnostics. Among the most understudied factors are DNA polymerases. In our study, we evaluated the effect of DNA polymerase type on the characterisation of bacterial communities, more precisely *Legionella*, using a genus-specific NGS approach. The assay with proof-reading high fidelity KAPA HiFi showed better amplification yield than the one with widely used non-proofreading HotStarTaq. *Legionella* community richness metrics were significantly overestimated with HotStarTaq. However, the choice of DNA polymerase did not significantly change the community profiling and composition. These results substantiate the use of proof-reading high fidelity DNA polymerases in NGS assays and highlight the need of considering the impact of different DNA polymerases in comparative studies and future guidelines for NGS-based diagnostic tools.

INTRODUCTION

Since its arrival, next-generation sequencing (NGS) has undergone fast and continuous progress in terms of technology, allowing enhanced performance, throughput and accuracy in a shorter time [1]. NGS methodologies have been widely applied in environmental microbiology to provide a broader characterisation of bacterial community structure, a better comprehension of the influence of environmental and anthropogenic drivers in both the emergence and persistence of bacterial pathogens; and for the detection and characterisation of clinically and environmentally relevant organisms [2–4]. One of the most environmentally and clinically important water-based bacterial taxa worth studying is *Legionell*a. *Legionella* species are persistently and abundantly found in a highly diverse set of aquatic environments and more than 25 species have been associated with disease [5]. Legionellosis outbreaks are frequently reported and have been mostly linked to hot water settings and foremost cooling towers, suggesting the need of a continuous environmental surveillance. NGS assays have already been demonstrated to have the capacity to provide a detailed understanding of the spatio-temporal dynamics and diversity of *Legionella* species and be used as reliable and precise monitoring tools of pathogenic species, such as *L. pneumophila* [6].

NGS has the potential to be an upgrade to the current accepted methodologies, and consequently be translated into active frameworks for routine surveillance and investigation of man-made freshwater systems. However, thorough *in vitro* and *in situ* validation studies are needed to evaluate if NGS-based methodologies are precise and reliable methodologies that are worth to be widely implemented and applied. Guidelines have already been presented and implemented for the use of NGS in clinical diagnostics and genetics screening [7–9]. Yet, no recommendations, guidelines or regulatory

71 approaches currently exist for validation and establishment of the assays and
72 interpretation of the results in environmental settings. Among the factors to consider in
73 the validation of a NGS assay are the potential bias introduced by library preparation and
74 sequencing steps, which can affect not only sensitivity and specificity of the molecular
75 assay developed but also inter-laboratory comparison studies. The potential of NGS as an
76 accurate and sensitive tool for environmental research and diagnostics can be affected by
77 the choice of the primers, targeted taxonomic group, PCR thermo-cycling conditions,
78 template concentration and DNA polymerase [10–13].

79 In this study, our aim was to evaluate the impact of the choice of the DNA
80 polymerase have on assessment of microbial diversity, more specifically on the alpha-
81 and beta-diversity of *Legionella* community in freshwater systems, with a genus-specific
82 16S rDNA-based NGS approach.

83

84 **MATERIALS AND METHODS**

85

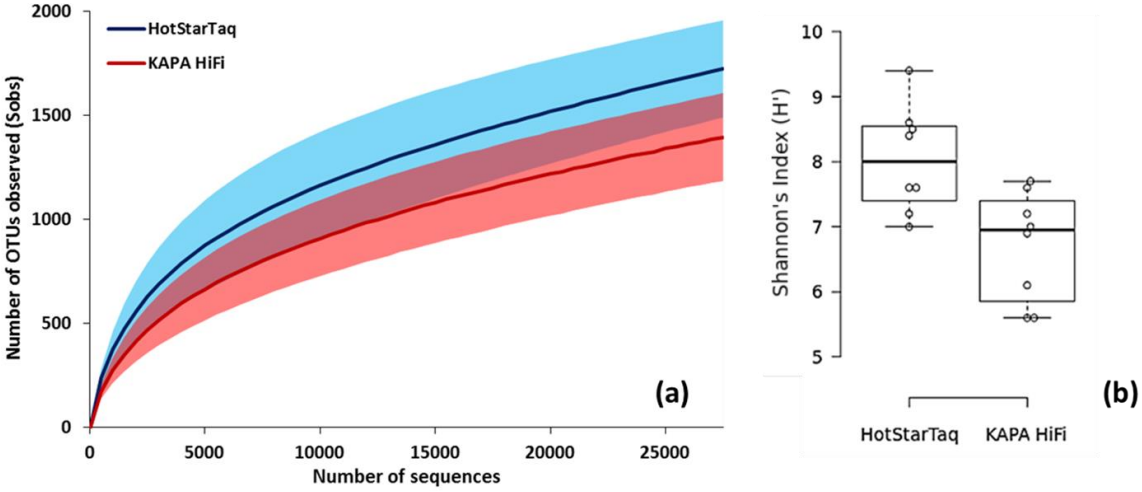
86 To study the effect of the DNA polymerase on *Legionella* libraries community
87 diversity metrics and structure, 8 freshwater samples, i.e. 2 cold drinking water (A and
88 B), 2 hot drinking water (C and D) and 4 cooling tower water (E to H) samples, were
89 collected at the campus of the Helmholtz Centre for Infection Research (HZI), in
90 Braunschweig, Germany, and processed as previously described [6,14,15]. These
91 freshwater samples were then analysed by a genus-specific NGS approach using two
92 optimised and independent assays with two hot-start DNA polymerases, HotStarTaq
93 (Qiagen, Hilden, Germany) and proofreading KAPA HiFi (KAPA Biosystems,
94 Wilmington, MA, USA). A 16S rRNA gene fragment, with a length of 421 bp, comprising
95 the V3-V4 hypervariable regions was amplified with *Legionella* genus-specific primer

pair Lgsp17F 5´-GGCCTACCAAGGCGACGATCG-3'/ Lgsp28R 5´-CACCGGAAATTCCACTACCCTCTC-3' [16]. Detailed descriptions of the used Illumina MiSeq-based approach with both DNA polymerases as well as of the 16S rDNA data processing and taxonomic classification by the bioinformatics pipeline of SILVA project are given elsewhere [17,18].

RESULTS

Before evaluating the effect of the DNA polymerase on community diversity metrics, we compared the yield of the two optimised assays by equivolume pooling of all sample libraries and sequencing in a single Illumina MiSeq 250 bp paired-end run. The data revealed a statistically significant difference between the sample sets concerning the number of retrieved 16S rRNA *Legionella* gene sequences per sample, after quality filtering and taxonomic assignment (Welch's t-test, $P<0.05$). The mean number of *Legionella* 16S rRNA gene sequences per sample in the sample set amplified by KAPA HiFi was significantly higher (52,385 ± 12,907) than in the one amplified by HotStarTaq (37,343 ± 8,995). Of the 8 samples, 7 had a higher number of sequences when amplified with KAPA HiFi, representing, on average, an increase of 45% when compared with HotStarTaq. This discrepancy does not seem to be an outcome of poor sequence quality as the sample sets presented a similar percentage of rejected sequences during quality filtering (1%). These results suggest that the optimised assay with KAPA HiFi polymerase provided a higher *Legionella* amplification yield than the one with HotStarTaq polymerase, regardless of the observed inferior specificity to *Legionella* genus (86% ± 11% against 97% ± 2%). Nonetheless, rarefaction analyses revealed

120 Operational Taxonomic Units (OTU) values levelling off markedly for all samples (data
121 not shown).
122



123

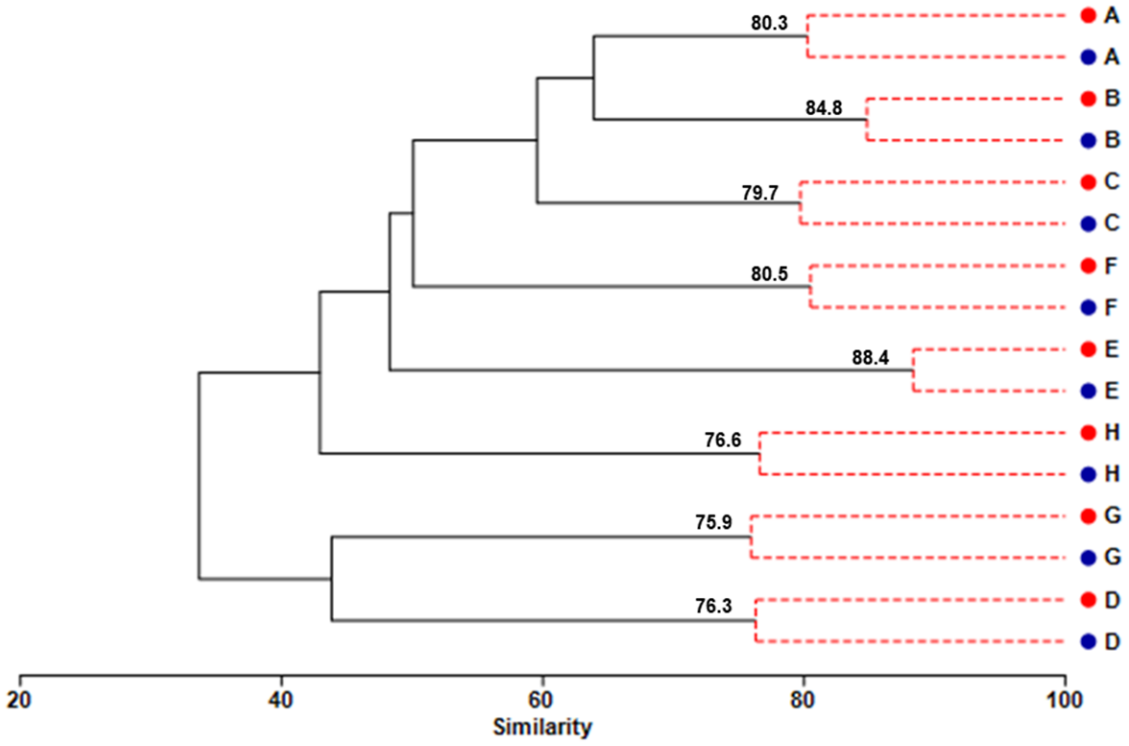
124 **Fig. 1. Alpha-diversity assessment of *Legionella* communities in 8 water samples**
125 **with HotStarTaq and KAPA HiFi DNA polymerases. (a)** Rarefaction curves showing
126 progress of number of OTUs observed with increasing number of sequences analysed.
127 Number of OTUs observed were calculated after normalisation to the sample with the
128 lowest number of sequences. Lines indicate mean values with corresponding coloured
129 shaded standard deviation for HotStarTaq DNA polymerase (blue) and KAPA HiFi DNA
130 polymerase (red). **(b)** Boxplots of Shannon's diversity index (*H'*) of 8 water samples
131 amplified with both DNA polymerases. Bars within boxes indicate the median value of
132 diversity. Whiskers extend to data points that are less than 1.5 x the interquartile range
133 away from $1^{st}/3^{rd}$ quartile.
134

135 Alpha-diversity metrics, i.e. observed OTU richness (OTU_{obs}) and Shannon's
136 diversity index (*H'*), were calculated, using the software package Explicet [19], for the
137 *Legionella* community of the 8 water samples amplified with both KAPA HiFi and
138 HotStarTaq DNA polymerases, after clustering of NGS reads into OTUs using an identity
139 criterion of 98%. (**Fig. 1**). The use of HotStarTaq, comparatively to KAPA HiFi, showed
140 a steeper slope and exhibited a higher *Legionella* richness with a statistically significant
141 increase of the number of OTUs observed (Welch's t-test, $p<0.05$) regardless of the

sample (**Fig. 1(a)**). The sample set when amplified by HotStarTaq had an averaged observed richness of 2389 ± 851 OTUs. However, when amplification performed with KAPA HiFi, the sample set showed an averaged observed richness of 1846 ± 469 OTUs, representing a decrease of 23% to the values of *Legionella* observed richness shown with HotStarTaq. Also, the number of singletons and doubletons were reduced by an average of 20% and 22%, respectively, with KAPA HiFi. Similar findings were observed when separately amplifying technical replicates (n=3) of a single strain (*L. pneumophila* ATCC 33152^{T}), with a total reduction of 38% in singletons and doubletons detected when using KAPA HiFi (data not shown).

Moreover, the comparison of the two sample sets showed a statistically significant difference in the Shannon´s diversity index (Welch's t-test, $P<0.05$) with higher values observed in samples amplified with HotStarTaq (**Fig. 1(b)**). Shannon´s diversity index on the samples amplified with HotStarTaq ranged from 7.06 to 9.94 (mean: 8.12 ± 0.97) whereas on the samples amplified with KAPA HiFi the index values ranged from 5.64 to 7.99 (mean: 6.86 ± 0.88).

To assess beta-diversity, we characterised the relative abundance and composition of the *Legionella* phylotypes in the freshwater samples amplified with each DNA polymerase (HotStarTaq and KAPA HiFi) and calculated the Bray-Curtis similarity, using PRIMER (Version 7.0.7) [20], to compare the level of community structure similarity between different samples and between the same sample amplified with the two different DNA polymerases (**Fig. 2**). The term *Legionella* phylotype is used according to what has been previously described [17]. Briefly, NGS sequences were assigned to a species when sequence identity ≥ 97%. The term phylotype englobes the acknowledged *Legionella* species as well as other defined sequence clusters with sequence identity <97% to a known species.



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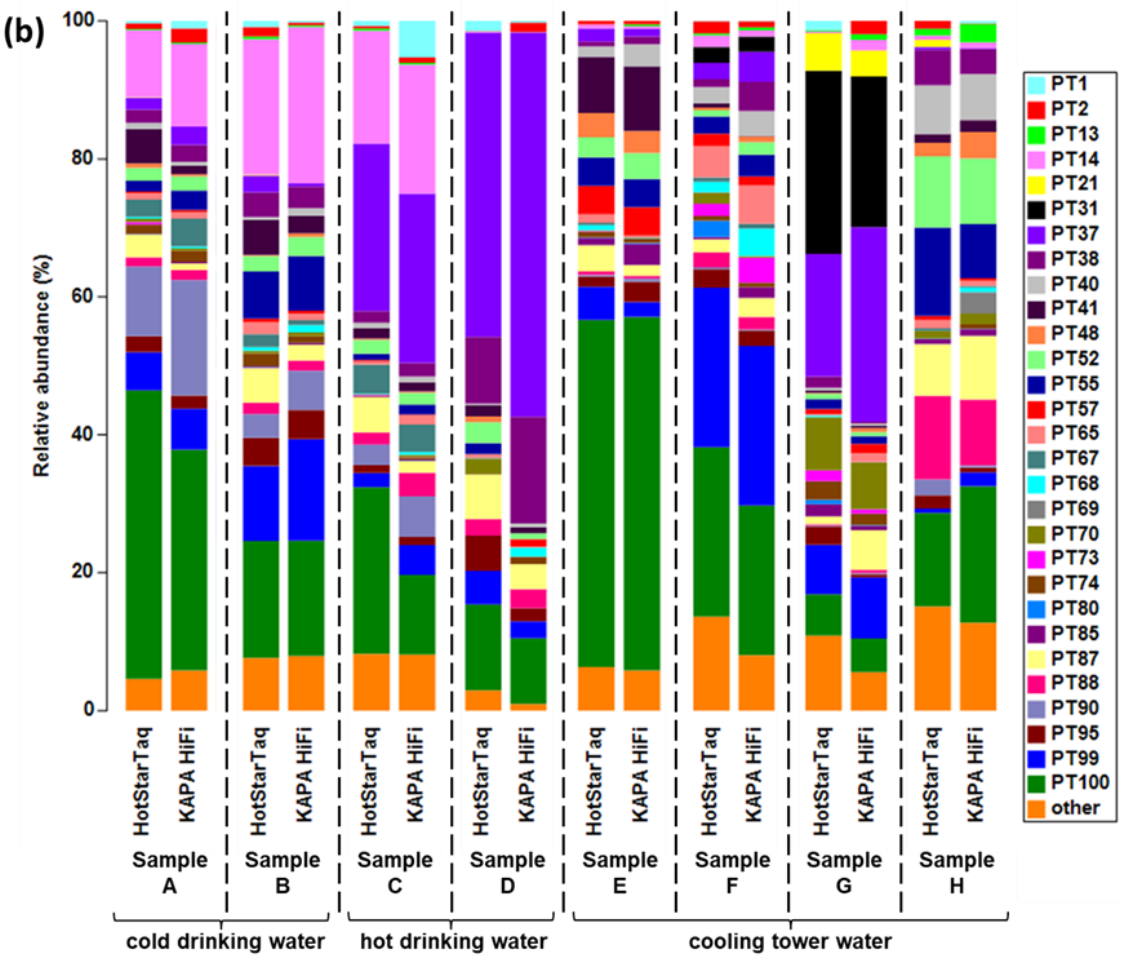

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Fig. 2. Effect of DNA polymerases on assessment of *Legionella* communities profiling. (a) Dendogram showing group-average hierarchical clustering of *Legionella* communities amplified in 8 water samples with both HotStarTaq (blue circle) and KAPA HiFi (red circle) DNA polymerases, using Bray-Curtis similarity**.** SIMPROF test was performed and red dashed lines in cluster analysis represent groups of samples that do not significantly differ in their *Legionella* community (P>0.05). Values on branches indicate Bray-Curtis similarity index. Sample A, cold drinking water March 2009; sample B, cold drinking water April 2009; sample C, hot water March 2009; sample D, hot water January 2014; sample E, cooling tower water April 2013; sample F, cooling tower water June 2013; sample G, cooling tower water August 2013; sample H, cooling tower water January 2015. **(b)** Bar graph representing relative abundances of *Legionella* phylotypes in 8 water samples amplified with both HotStarTaq and KAPA HiFi DNA polymerase. Phylotypes labelled from 1 to 52 correspond to described *Legionella* species. PT1, L. adelaidensis; PT2, *L. anisa*; PT13, *L. drozanskii*; PT14, *L. dumofii*; PT21, *L. gratiana*; PT31, *L. maceachernii*; PT37, *L. pneumophila*; PT38, *L. quateirensis*; PT40, *L. rowbothamii*; PT41, *L. rubrilucens*; PT48, *L. tucsonensis*; PT52, *L. worsleiensis*. Different freshwater samples are separated by a dashed line.

Based on SIMPROF analysis (**Fig. 2(a)**) [21], we observed that the difference introduced in the *Legionella* community structure by the use of the two different enzymes was not statistically significant in the sample set used (SIMPROF, P>0.05).

Nonetheless, shifts in the *Legionella* community of every sample studied were observed (**Fig. 2(b)**), with the similarity levels ranging from 75.9 (Sample G) to 88.4 (Sample E), with an averaged value of 80.3 ± 4.4. Clear clusters for every sample, independently of the type of water, were formed and unambiguously separated the 8 different water samples tested. Furthermore, analysis of the composition of the *Legionella* community in each sample amplified with the two DNA polymerases, revealed a substantial overlap of *Legionella* phylotypes. When the composition of the communities amplified by the two enzymes were compared, 7 out of 8 samples shared more than 85%

198 of the phylotypes (data not shown). Sample D showed the lowest percentage of shared
199 phylotypes (60.1%).
200
201 **DISCUSSION**
202
203 Currently, NGS technologies are widely applied in the characterisation of
204 communities, discovery of novel microorganisms and pathogen detection [22]. For NGS
205 assays to succeed on these fields, a high amplification efficiency during library
206 preparation and target enrichment is of critical importance to downstream sequencing,
207 especially when targeting specific low-abundant taxa in complex environmental samples.
208 The amplification efficiency is influenced by several PCR-related factors, including the
209 choice of DNA polymerase. Our results, despite a better performance of KAPA HiFi,
210 confirmed that the two different polymerases had a very good amplification performance
211 allowing a subsequent appropriate sequencing depth of the target. This was in a certain
212 amount expected due to the hot-start nature of the two enzymes. Plus, our findings
213 highlight that the choice of DNA polymerase, as well as optimisation of the cycling
214 parameters in PCR-based NGS assays, is important to find an appropriate balance
215 between non-specific amplification and yield, in order to maximise detection/sensitivity.
216 In addition, several studies have reported the impact of different amplification and
217 sequencing-related factors on high-throughput studies of microbial communities and how
218 these might distort diversity estimates and community profiling [23–25]. Yet, only a few
219 studies have evaluated the effects of DNA polymerases on the study of communities [26–
220 29].
221 Past NGS studies have revealed increasing richness values with increasing
222 sequencing depth [17,30]. Yet, our results showed that the freshwater samples amplified

by HotStarTaq DNA polymerase, despite a lower sequencing depth, had significantly higher OTU-based alpha-diversity metrics than KAPA HiFi DNA polymerase, indicating that alpha-diversity measurements are enzyme-dependent, as previously suggested [26–29].

A recent study by Pereira et al. [17], using the same approach, revealed that when comparing the sequence accuracy and quality of the generated NGS *Legionella* libraries, KAPA HiFi had a significantly higher fidelity, with a mean error rate of 0.38% against 0.45% and 65% of error-free sequences against 39%. All data considered, the significant increase in bacterial community richness and diversity, plus the significant higher number of singletons and doubletons observed after amplification of the same 16S rRNA region with HotStarTaq DNA polymerase, is almost certainly linked to a higher generation of erroneous sequences with an enzyme without proofreading activity. This leads to an increased number of OTUs and consequently an artificial overestimation of the richness and diversity estimators [23,31,32]. Similar inflated community richness with polymerases presenting lower fidelity was observed in bacterial libraries by Wu et al. [26] and fungal libraries by Oliver et al. [28]. Yet, conflicting reports by Ahn et al. [27] and Qiu et al. [29] have detected an overestimation of richness with a high-fidelity enzyme instead, attributing it to a higher frequency of chimeric sequences. In our study, as we are targeting a specific taxa, low diversity libraries are generated, not allowing to precisely quantify the potential amount of chimeric sequences in the water samples. However, analysis of *Legionella* mock communities with the same NGS approach has revealed a small representation of these spurious sequences with KAPA HiFi [17].

When comparing beta-diversity metrics after amplification with both DNA polymerases, the impact of the enzymes on the *Legionella* community composition and structure (beta-diversity) was not significant for the samples studied, contrarily to what

was indicated by Wu et al. [26], when comparing the DNA polymerases TAKARA ExTaq with PfuUltra II Fusion HS. Though HotStarTaq and KAPAHiFi have different properties, kinetics and slightly different polymerase cocktails were used, the two enzymes seem to have an alike amplification profile of the different *Legionella* phylotypes. However, differences in Bray-Curtis similarity between the samples amplified by two distinct DNA polymerases were observed (mean BC: 80.3 ± 4.4). These discrepancies on *Legionella* community profiling are slightly higher to the ones previously observed between technical replicates (mean BC: 86.8 ± 4.9) [17].

In summary, our data highlights the potential advantageous effects of the use of proof-reading high-fidelity enzymes such as KAPA HiFi on NGS library preparation methodologies. As well, this study emphasises the influence the choice of DNA polymerase has on the characterisation of microbial communities, especially in alpha-diversity metrics, and the critical importance of taking this factor in consideration in comparative studies and in the future use of these high-throughput technologies for pathogen detection and quantification.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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