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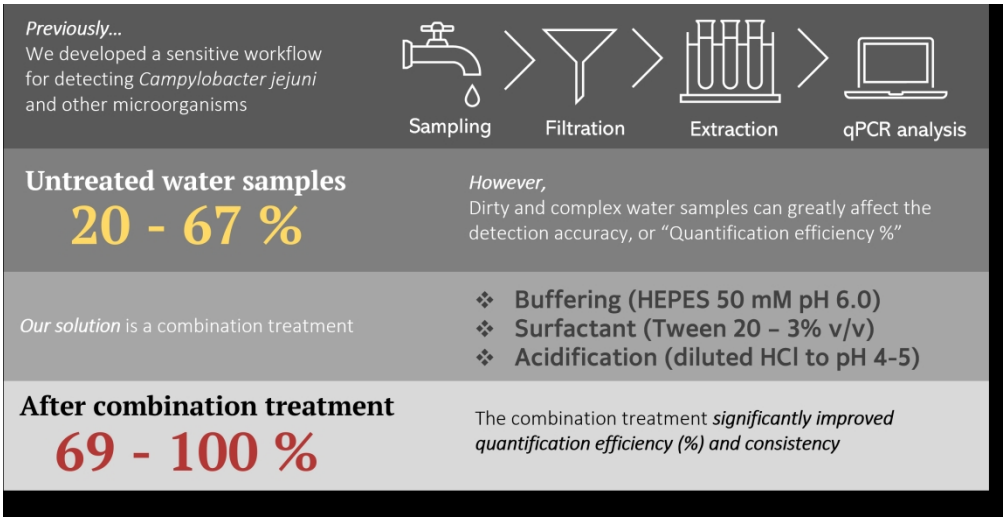

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Adapted Method for Rapid Detection and Quantification of Pathogen Campylobacter jejuni from Environmental Water Samples

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Abstract

Building on a previously developed workflow for rapid and sensitive pathogen detection by qPCR, this work has established a sample treatment strategy that produces consistent quantification efficiencies (QEs) for *Campylobacter jejuni* against a complex and highly variable sample matrix from a suburban river. The individual treatments most effective at minimising the inhibitory effects of the sample matrix were pH buffering with HEPES (50 mM, pH 5.7) and addition of the surfactant Tween 20 (2% v/v). Unexpectedly, sample acidification (pH 4–5) resulting from the use of aged Tween 20 that had undergone partial hydrolysis, appeared to play a key role in enhancing QE. This effect could be replicated by direct pH adjustment with dilute hydrochloric acid and may be linked to the solubilisation and removal of inhibitory particles at an acidic pH. While the effectiveness of each individual treatment method varied, a combined treatment of either HEPES buffer + Tween 20, or direct pH adjustment + Tween 20, consistently produced QEs of 60–70% and up to 100%, respectively, over a sampling period of one year. The consistency and scalability of this workflow make it a suitable alternative to culture-based ISO methods for detecting *Campylobacter* spp.

Keywords

qPCR; waterborne pathogen; filtration; *Campylobacter jejuni*; water testing; environmental water

1. Introduction

Campylobacters are one of the leading causes of waterborne diseases worldwide and are commonly associated with bacterial diarrhoea illness in humans (Australian Government Department of Health 2019). In developed countries such as Australia and New Zealand, where stringent water regulations are in place, the *Campylobacter* spp. are frequently reported as causing the greatest burden of disease and incidence rates amongst waterborne pathogens (Gibney et al. 2017; Ministry for the Environment - New Zealand Government 2007).

Early detection is one of the important strategies in preventing and containing diseases from *Campylobacter*, and it is traditionally carried out using a standardised method involving culture-based selection and direct colony counting (International Organization of Standardization 2019). While the conventional quantification method has been proven to be reliable in detecting *Campylobacters*, field application and timely reporting may be hindered by long incubation (48–72 h), labour-intensive enumeration and the requirement for heavy or specialised laboratory instruments. Furthermore, quantification accuracy of a culture-based method may be compromised by the presence of viable but nonculturable (VBNC) cells, preventing proper assessment of the *Campylobacter* risks in water (Lv et al. 2020).

We have previously established an enzyme-based workflow that addresses the limitations listed above. The quantification workflow utilises the prepGEM extraction system, based on an efficient and thermostable proteinase isolated from *Bacillus* spp., directly compatible with PCR, qPCR and NGS procedures (Boykin et al. 2019; Holmes and Roman and Hughes-Stamm 2018; Lounsbury et al. 2012; Stanton et al. 2019). The enzyme and molecular based workflow bypasses lengthy incubations and can be performed using portable equipment, making it well suited for rapid screening in the field (Boykin et al. 2019; Saul et al. 1996; Stanton et al. 2019). With some protocol modifications, it was possible to achieve quantification sensitivity and accuracy comparable to, if not exceeding the conventional methods (Sun et al. 2021).

Environmental and drinking water samples are complex and their composition can vary significantly due to weather and human/animal activities. Inhibitors contained in these samples have been found to interfere with nucleic acid extraction and quantification (Leblanc-Maridor et al. 2011; Ricke et al. 2019). While existing solutions, such as column-based filtration and magnetic bead separation, help to remove inhibitors, they also result in sample loss, which compromises quantification sensitivity (Josefsen et al. 2010; Lund et al.

2004; Schnider et al. 2010). A purification strategy that could effectively remove most of the inhibitors without compromising DNA yield and qPCR sensitivity is crucial in developing a universal solution for environmental water monitoring.

This work aims to adapt and refine the established enzyme-based DNA extraction and quantification workflow (Sun et al. 2021), so that it can be applied to complex and variable sample matrices and produce reliable results without compromising sensitivity, paving the way for a universal water monitoring solution.

2. Materials and Methods

2.1 Pathogen Capture and gDNA Extraction

River water (RW) samples were collected from Kikkiya Creek, Sydney, NSW Australia (GPS coordinates: −33.774863, 151.118338). For experimental controls, MilliQ-H_2O was used as the water matrix. The water samples were spiked with *Campylobacter jejuni* (IFM 2454) cells, stock obtained from IFM Quality Services Pty Ltd, Ingleburn, AU, and maintained on Sheep Blood Agar plates (Thermo-Fisher Scientific, AU) in a microaerobic environment until use. Upon collection, the water samples were spiked with *C. jejuni* cells to give approximately 300-1000 cells per qPCR reaction. Filter-capture of the pathogen was performed by passing the spiked water samples through a 25 mm Swinnex-Filter unit with a 0.4 μm polycarbonate filter of 10 μm thickness (HTTP02500, Merck-Millipore, AU). Thicker polycarbonate filters require more extraction solution and thus should be avoided where possible.

Extraction of the gDNA of *C. jejuni* cells was performed using the prepGEM Bacteria kit (MicroGEM, NZ) with a modified protocol for filter membranes. The filters were squeezed with tweezers to fit tightly into the bottom of a 1.5 mL microcentrifuge tube, to which the extraction mixture was added (90 μL DNA-free water, 10 μL 10× GREEN+ Buffer, 1 μL prepGEM enzyme). Digestion was performed at 75 °C for 15 min followed by enzyme inactivation at 95 °C for 5 min. Extracted DNA was used immediately for quantification via qPCR, or stored at −20 °C.

2.2 Quantification via Real-Time Quantitative PCR (qPCR)

Quantification of the *C. jejuni* cells was performed via qPCR as described previously (Sun et al. 2021). The primer pair HipO-F (5′ TGCTAGTGAGGTTGCAAAAGAATT 3′, 250 nM final concentration) and HipO-R (5′ TCATTTCGCAAAAAAATCCAAA 3′, 100 nM final concentration) were used to amplify *C. jejuni* gDNA.

qPCR reactions were prepared in triplicate and consisted of 10 µL qPCR of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, AU), 2 µL each of Forward and Reverse primers and 6 µL of the *C. jejuni* gDNA extract. Real-time PCR was performed on a LightCycler 480-II (Roche, US) using the following thermocycling program: 1) UDG activation 50 °C, 2 min; 2) Dual-Lock DNA polymerase 95 °C, 2 min; 3) Quantification (× 40 cycles) - Denature 95 °C, 15 s followed by Anneal/Extend 60 °C, 1 min.
Following qPCR, the crossing point (Cp) values for each sample were converted to number of cells using the established standard curve for *C. jejuni* (Sun et al. 2021). The quantification efficiency (QE%) representing the efficiency of quantification for this workflow was determined by comparing the difference between spiked cell input and estimated cell count determined from the qPCR Cp and calculated as: Quantification efficiency % (QE) = Number of cells detected using this workflow / Number of cells spiked × 100%.

2.3 Sample Treatment Processes

2.3.1 Centrifugation and Filtration Treatments

For centrifugation treatments, RW samples were collected and centrifuged at 17,000 rcf for 10 min prior to cell spiking and applying the quantification workflow.
Filtration treatment was performed after the gDNA extraction step; the gDNA extract was passed through a 0.2 µm polycarbonate filter (GTTP02500, Merck-Millipore, AU) to remove contaminating particulates, and the flow-through containing gDNA collected for quantification.

2.3.2 Tris, HEPES and MOPS Buffered Extraction Mixture

Prior to gDNA extraction, the prepGEM extraction mixture was prepared as described in Section 2.1, followed by the addition of 1 M buffer stocks to achieve a final concentration of 20, 50 or 100 mM. All buffers were prepared fresh on a weekly basis and stored away from light at 4 °C.
To prepare 1 M Tris buffer, 1.21 g of Tris base was dissolved in 7.5 mL of MilliQ-H_2O, followed by adjustment to pH 6.9 with concentrated HCl before filling with MilliQ-H_2O to a final volume of 10 mL. To prepare 1 M HEPES buffer, 2.38 g of HEPES was dissolved in 7.5 mL of MilliQ-H_2O and adjusted to desired pH (pH 5.3–7.2) with 10 M NaOH before filling with MilliQ-H_2O to a final volume of 10 mL. For MOPS buffer, 2.09 g of MOPS was dissolved in 7.5 mL of MilliQ-H_2O, adjusted to desired pH (pH 5.9–6.6) with 10 M NaOH and filled to 10 mL with MilliQ-H_2O.

2.3.3 Filter Treatment with Sulfuric Acid and Sodium Hydroxide Solutions

Acid (sulfuric acid) and alkaline (sodium hydroxide) washes were performed on the Swinnex filter units after the RW filter capture step. Concentrated sulfuric acid (98%) was diluted to the appropriate concentrations (0.25–1.25% v/v) by carefully pouring into MilliQ-H_2O. Sodium hydroxide stock (10 M) was prepared and diluted to 25 mM immediately before use. For each filter to be washed, 10 mL of diluted acid/alkaline solution was slowly passed through the Swinnex filter unit, followed by rinsing with 5 mL of MilliQ-H_2O. Each wash and rinse step involved a purging process where some air was injected into the unit through the syringe to remove any residual reagents from the filter. The rinsed filters were used directly for gDNA extraction.

2.3.4 Tween 20 and Tween 80 Treatment

Tween 20 and Tween 80 stocks were sourced from various suppliers, as detailed in Table 1. The year of manufacture and percent hydrolysis (as determined by NMR spectroscopy) are specified as these parameters are pertinent to later discussions. The spiked water samples were treated with Tween 20 or Tween 80 to the appropriate final concentration (up to 3% v/v; for Tween 20 and up to 8% v/v for Tween 80) before the filter capture process. Samples containing Tween 20/Tween 80 were inverted gently to minimise foaming. The adjustment of water sample pH after the addition of Tween 20 was performed with dilute hydrochloric acid (10% v/v or 1 M).

*** Table 1 to go near here ***

2.4 NMR spectrometry

NMR analyses were performed using a Bruker Avance III HD 400 MHz NMR spectrometer equipped with a 5 mm BBFO SmartProbe. For all analyses, 10 mg of sample was dissolved in 500 µL of DMSO-d_6 in a 5 mm Pyrex tube. Spectra were referenced to residual non-deuterated solvent signals (d_H 2.49; d_C 39.5).

2.5 De-esterification of Tween 20 by acid-catalysed methanolysis

3 drops of concentrated H_2SO_4 were added to a solution of Tween 20 (1.0 g) in MeOH (16.0 mL). The reaction mixture was refluxed for 27 h, cooled to room temperature and the solvent was removed *in vacuo* at 40 °C. The oily residue was triturated vigorously with hexane (4 ×

10 mL; 3 min each) then dried under high vacuum for 16 h to afford the de-esterified Tween 20 product as a pale-yellow oil (0.87 g, quant.). List of parameters: ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 4.53 (dd, J = 4.5 Hz, 1H), 4.41 (d, J = 4.5 Hz, 1H), 3.98 (m, 1H), 3.92 (m, 1H), 3.53-3.48 (m, 72H), 3.47 (br t, 8H), 3.40 (br t, 8H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 85.5, 84.0, 79.8, 79.7, 72.3, 69.8, 60.2.

3. Results and Discussion

3.1 Preliminary trials for inhibitor removal from river water samples

To represent a complex and variable environmental water matrix, samples from a small river with running water (Kikkiya Creek, Sydney, Australia) were collected and tested using our previously established pathogen quantification method (Sun et al. 2021), over the course of one year. The river water (RW) samples were found to be moderately inhibitory, preventing accurate quantification of the spiked-in *C. jejuni* cells. The extent of the inhibitory effect was represented in terms of quantification efficiency (QE), calculated as described in materials and methods Section 2.2. In this work, QE is defined as the percentage of cells quantified by our method in comparison to the total quantity of cells spiked into the sample. The QE level of the untreated RW fluctuated daily (20–67%, Table 2- Untreated samples) possibly due to changing levels of inhibitors linked to environmental factors including season, water level, and anthropogenic processes (observations, data not shown).

Several preliminary treatment strategies were trialled to remove or reduce the impact of the inhibitors, with the results shown in Table 2. Some inhibitors may be solubilised by an acid or alkaline wash (Table 2; sulfuric acid and NaOH wash), while insoluble particles can be removed by centrifugation or pre-filtration (Table 2; Centrifugation and Filtration). Increasing the amounts of PrepGEM enzyme, a type of protease, may improve QE (Table 2; PrepGEM enzyme 1 and 4 µL) if the water sample contains protease inhibitors. If fluctuations in pH inhibit DNA extraction or qPCR, then a pH buffer may be an effective treatment (Table 2; MOPS, HEPES and Tris buffers). Furthermore, to overcome possible autoagglutination and adhesion of *C. jejuni* cells to vessel surfaces (Golden and Acheson 2002; Reuter et al. 2015), which interferes with the filter quantification process, the samples were treated with surfactant Tween 20 (Table 2; Tween 20, 0.25–2%).

*** Table 2 to go near here ***

Based on the preliminary trial results, the use of Tris buffer, HEPES buffer and Tween 20 supplementation were found to be the most effective treatments for improving QE. These buffer-treated samples resulted in QEs ranging from 48% (Table 2; HEPES buffer pH 7.2, 50 mM) to 64% (Table 2; Tris buffer pH 6.9, 20 mM and 50 mM), which is an improvement from untreated samples tested in the trial experiment (QE 20–67%). Further testing revealed that the enhanced QEs observed from the buffer treatments (Tris and HEPES) were correlated with pH of the solution (Figure 1), with the highest QE (86%) obtained when the PrepGEM extraction buffer was supplemented with HEPES buffer (50 mM) at pH 6.0. This finding suggests that the native pH of the untreated samples (Figure 1; RW Untreated, around pH 8.0) may be sufficiently alkaline to interfere with the downstream extraction and qPCR process. The alkaline samples could be neutralised by the addition of buffers at an acidic pH (pH 6.0).

The decision to utilize HEPES buffer also considered the buffering range of the Tris and HEPES buffers. It was observed that sample extraction appeared to be more efficient at an acidic pH, which is only achievable using the HEPES buffer (refer to Figure 1 and 5 and the discussion for further information). Although Tris buffer demonstrated an ability to improve QE, it was not considered an ideal candidate due to its effective pH range (pH 7–9) being too high for the acidic pH (pH 6.0) required for sample treatment.

*** Fig. 1 to go near here ***

Aside from pH buffering, the addition of a surfactant (Tween 20) was shown to be effective in the trial, resulting in QEs of up to 63% (Table 2; Tween 20 0.5–2% v/v). Further testing of Tween 20 at a range of concentrations (0.25–3% v/v) revealed the greatest enhancement in QE (77%) was observed at 3% v/v (Figure 2). The related surfactant Tween 80 achieved a similar enhancement in QE (68%), albeit at a higher concentration of 6% v/v (Figure 2). The enhancing effect of surfactants may be due to their ability to solubilise lipids and other hydrophobic compounds that inhibit DNA extraction or qPCR. They could also help prevent cell aggregation and adhesion to the sample containers. Based on the findings in the preliminary results, HEPES buffer at pH 5.7, 50 mM, and Tween 20 at 3% v/v were found to be optimal for water sample treatment.

*** Fig. 2 to go near here ***

Several methods listed in Table 2 yielded suboptimal results, however, valuable insights could be gained from these observations. Centrifugation, for instance, had led to a decrease in (QE) since it not only eliminated impurities from the water sample, but also pelleted bacterial cells. Even at a low rcf, the bacteria sedimented at a similar pace as the impurities, rendering it impossible to exclusively eliminate contaminant particles while retaining bacteria within the water sample for quantification purposes.

The acid and alkaline wash (Sulfuric acid and NaOH wash, Table 2) yielded low QEs (<40%). While we initially explored this method due to its success in our previous tap water treatment study (Sun et al. 2021), it has proven to be a two-edged sword. While it has the potential to remove insoluble particles that cause membrane blockages, it also poses a risk of solubilizing cells and removing them from quantification. Therefore, this method requires careful assessment on a case-by-case basis. Our findings indicate that the potential benefits of acid/alkaline washes are outweighed by the negative impact on cell quantity. Consequently, the use of this method was excluded from the workflow.

3.2 Modification of the Tween 20 treatment for optimal QE

While the addition of Tween 20 was found to produce the highest QE, its effectiveness appeared to be dependent on the age of the reagent. A comparison of Tween 20 reagents of different ages revealed that the QE-enhancing effect was most significant with Tween 20 that had been in storage for long periods (Figure 3; T20-2005-AMRESCO, 16 years old, QE 87%; T20-2006-Sigma, 15 years old, QE 84%). This suggested that the observed enhancements in QE may be due to the presence of decomposition products or other impurities in the Tween 20 samples, in addition to Tween 20 itself.

*** Fig. 3 to go near here ***

Tween 20 is a nonionic surfactant consisting of a polyethoxylated sorbitan moiety esterified at one hydroxy terminus with lauric acid (or oleic acid in the case of Tween 80). The degradation of Tween 20 under a variety of conditions has been studied extensively, with the two major degradation pathways being autooxidation and hydrolysis (Kerwin 2008; Li et al. 2014; Martos et al. 2017). Autooxidation occurs in the presence of atmospheric oxygen and is accelerated by light, heat and certain metal ions. Autooxidation generates reactive hydroperoxides, which result in side-chain cleavage and the production of species such as hydrogen peroxide, formic acid, formaldehyde and acetic acid, amongst others. Hydrolysis of

the lauric acid ester of Tween 20 is also known to occur on long-term standing at room temperature and is accelerated under acidic or basic conditions and at elevated temperatures. The purity of each of the Tween 20 reagents was evaluated by ^{1}H NMR spectroscopy in DMSO-d_6 (Figure 4). While all reagents were found to contain trace levels of a variety of impurities, the main source of degradation appeared to be due to hydrolysis of the laurate ester. The methylene protons alpha to the carbonyl group were well-resolved in the ^{1}H NMR spectra, resonating at d_H 2.27 as the laurate ester and at d_H 2.17 as the free acid. This allowed the ratio of peak areas to be used to determine the percentage of Tween 20 that had undergone hydrolysis in each sample (Table 1). The degree of hydrolysis was also found to be correlated with sample age, ranging from < 1% hydrolysis for the 2021-Sigma reagent, through to 24% hydrolysis for the 2006-Sigma reagent. Therefore, it was concluded that either the de-esterified polyethylene sorbitan component of Tween 20 or the liberated free lauric acid was responsible for the observed improvements in QE.

*** Fig. 4 to go near here ***

To determine the effects of each individual Tween 20 degradation product on enhancement of QE, we first synthesised a sample of the de-esterified polyethylene sorbitan component of Tween 20 by acid-catalysed methanolysis of fresh T20-2021-Sigma stock (Figure 5). Significantly, the sorbitan component resulted in a lower QE than observed for fresh Tween 20 (Figure 3; De-esterified T20 29% vs. T20-2021-Sigma 42%), suggesting it is unlikely to be the main contributor to the enhanced QE. It was not possible to directly test the lauric acid component of Tween 20 since it does not dissolve in water in its fatty acid form. Although its sodium salt (sodium laurate) was water-soluble, it caused issue by forming precipitates with the RW samples being tested, which blocked the filter membranes. However, we were able to indirectly assess the effect of the lauric acid component by comparing the pH changes between the various Tween 20 samples. The aged Tween 20 reagents were observed to have a strong acidifying effect on the RW samples (pH 6–7 prior to, and pH 4–5 after the addition of the aged Tween 20, data not shown). This effect could be emulated by adjusting the pH of a solution of fresh Tween 20 with dilute hydrochloric acid. As shown in Figure 6, the QE observed using aged Tween 20 and pH-adjusted fresh Tween 20 were very similar (S5: ~100% for both; S6: 65% vs. 69%). This suggests that the acidic pH resulting from Tween 20 degradation is the main contributor to the enhanced QE observed.

*** Fig. 5 to go near here ***

The link between acidic pH and high QE was not further investigated in this work. However, it has been established in our previous report that a phosphoric acid wash of the filter membrane helps to remove insoluble salts, such as $CaCO_3$ and $MgCO_3$, which can block the filter membrane and inhibit downstream quantification (Sun et al. 2021). While the on-filter acid wash did not enhance the QE of RW samples (sulfuric acid wash – Table 2; phosphoric acid wash – data not shown), a sample acidification process with hydrochloric acid (pH 4–5) produced marked improvement of QE (Figure 6; S5 and S6, QE 69–100% after pH adjustment). This suggests the presence of other types of insoluble particles in RW that could be removed with a lower pH or hydrochloric acid.

3.3 Achieving consistent QE results through simple combined treatments

One of the main aims for this work was to establish a quantitative workflow that produces consistent results using environmental samples, the composition of which can vary widely due to seasonal and anthropogenic factors. Therefore, following identification of Tween 20, HEPES buffer and direct pH adjustment as the most effective treatments for QE, we moved to assess their general effectiveness against a wide range of RW samples.

To ensure sample representativeness, RW was collected under different weather conditions and water levels (dry riverbed, high water, rainy and sunny days) over the course of one year. The samples were then treated with a combined method (detergent plus pH-based treatment) of either a) Tween 20 (3% v/v) and HEPES buffer pH 5.7, or b) Tween 20 (3% v/v) with direct pH adjustment (pH 4–5).

The combined methods seemed to consistently produce a significant improvement on QE (Figure 6; combined treatment, navy and dark green columns, QE 55–90%), in contrast with the HEPES buffer or T20 only methods (Figure 5, light and dark blue columns), which yielded lower QEs with high variability (QE 7–65%). An exception was seen for one pH-adjusted sample (Figure 5; S5, old T20 only, pH 4.99, 103%). These results suggest that consistent QEs can be achieved via a combination of compatible treatments, drawing on the strengths of each individual treatment that is effective against specific types of inhibitors.

In addition to consistency, the QEs achieved in this work were also notably higher than typically observed for similar qPCR-based environmental *C. jejuni* detection methods (2.0 +/- 0.8%) (Tissier et al. 2012), and much simpler compared to other sensitive workflows that require elaborate enrichment steps (3 CFU per 100 mL detected, requiring culture enrichment

and semi-nested PCR assay (Oyofo and Rollins 1993; Ricchi et al. 2017; Russo et al. 2014; Waage et al. 1999). The reduced capture and detection times enabled by our method could potentially contribute to faster response and remediation in environmental monitoring applications.

3.4 Sample scaling up to 100 mL without affecting QE

This workflow has been tested at 10, 40, 80 and 100 mL, without significantly compromising QE (Figure 5). At 100 mL, the sample volume is on par with typical volumes used for bacterial detection in environmental water (Australian Standard AS/NZS 2016; International Organization of Standardization 2019), and thus is suitable for field application without further adjustment. Partial filter blockage occurred with sample volumes larger than 100 mL, requiring pressures higher than achievable by manual syringe filtration (observation, data not shown). Environmental water typically contains particulates that can block filter membranes, which may be addressed by pre-filtration or the use of a larger pore filter. However, as previously assessed, both pre-filtration and use of membranes with larger pores (> 0.6 μm) resulted in cell loss and thus compromised QE (Sun et al. 2021). A compromise, albeit at a slightly higher cost (extra filter membranes, approximately $1 USD each), was to process large sample volumes with multiple 100 mL filtrations as described in Section 2.1. Two thin polycarbonate filters (< 10 μM thickness, 25 mm diameter, HTTP02500, Merck-Millipore, AU) can be processed per 100 μL prepGEM reaction, which helps to reduce cost per sample.

3.5 Practical considerations for field application – pH adjustment

Although the combined method using direct pH adjustment (Figure 5; S5 and S6, QE 69–100%) outperformed the combined method with HEPES buffer (Figure 5; S1-4, QE 55–90%), the former can be difficult to perform in the field due to extreme pH changes in a non-buffered solution. Additionally, direct pH adjustment requires a pH electrode and hydrochloric acid, which adds a potential hazard. Care must be taken while utilising HCl for pH adjustment, as excessive acidity could lyse the cell or weaken the polycarbonate filter membrane (at 20% v/v hydrochloric acid, (CP Lab Safety 2021)). It is therefore suggested that the HEPES buffer be used in a field setting due to its ease of application while still achieving consistent outcomes. Alternatively, an acidic pH may be monitored by using litmus test strips and carefully adjusting with dilute hydrochloric acid.

*** Fig. 6 to go near here ***

4.1 Conclusion

Building on the previously established rapid and sensitive enzyme-based pathogen detection workflow against tap water, this study further demonstrated that the qPCR detection method could be adapted for use on environmental water samples of highly variable composition. A sample treatment method consisting of pH buffering with HEPES buffer, and the addition of detergent Tween 20 or Tween 80, had been shown to consistently produce quantification efficiencies in the range of 55–90%), a significant improvement from the untreated controls. This workflow was tested in sample volumes up to 100 mL, in line with commercial standards for water pathogen testing against *C. jejuni* spp. The quantification efficiency was further improved up to 100% when the pH of the water sample is adjusted to pH 4–5, although adaptation may be required for practical application in the field. With a sampling time of only 30 minutes (10-minute filtration and 20-minute extraction), the rapid nature of this method makes it an attractive alternative to the overnight culture-based ISO methods used for detecting *Campylobacter* spp. and other pathogenic microorganisms.

4.2 Recommended protocol for *Campylobacter jejuni* quantification

This is the modified workflow for quantifying *Campylobacter jejuni* in environmental water samples with insoluble particulates, building upon our previous methodology to accommodate the increased variability in composition due to seasonal changes, weather conditions, and animal activities.

1. **Filtration** – Water samples up to 100 mL filtered through one 25 mm Swinnex adapter with a polycarbonate filter (10 µm thickness, 25 mm diameter, 0.4 µm pore size, HTTP02500, Merck-Millipore, AU) using a syringe or pump. Multiple filters may be required for sample volume up to if blockage occurs. Centrifugation may be used as a pre-treatment for samples with high concentrations of large particulates, but this method should otherwise be avoided as it removed cells from the quantification process (Section 3.1). Acid wash (10 mL of 20% (v/v) phosphoric acid) was beneficial for samples with higher mineral content, but can cause cell loss due to cell solubilization (Sun et al. 2021). In the present study, both acid and alkaline washes were found to have a detrimental effect on overall quantitative efficiency (QE%) for the river water sample and was excluded from the workflow. The effects of acid and alkaline washes should be assessed for different field samples.

2. **Sample treatment and DNA extraction** – A 100 µL reagent mixture containing the DNA extraction enzyme, HEPES buffer pH 5.7, 50 mM and Tween 20 at 3% (v/v) was prepared (5 µL of 1M HEPES buffer pH 5.7, 10 µL of Tween 20 stock at 30% v/v, 75 µL DNA-free water, 10 µL 10× GREEN+ Buffer, 1 µL prepGEM enzyme) and added to the filter to condition the samples for DNA extraction. Tween 20 provided the best QE%, but performance could vary due to age of the reagent (Section 3.2). Tween 80 at 6% (v/v) may be substituted for a more stable performance but at the cost of lower QE and higher reagent usage.
3. **DNA extraction** – Filters were removed carefully from the Swinnex adapter, folded and squashed to fit into the bottom of an Eppendorf tube so it could be totally submerged in the 100 µL reagent mix prepared in Step 2. While multiple filters could be fitted into one Eppendorf tube for, it is recommended that no more than two filters (from 200 ml of samples) be used for per reaction, as the insoluble particles from samples could interfere with extraction reagent. The mixture was then incubated at 75 °C for 15 min for digestion, and 95 °C for 5 min for enzyme inactivation.
4. **Quantification** – Quantification via qPCR could be performed immediately following the DNA extraction without further treatment. Thermocycling and subsequent quantitative analysis performed as detailed in the methods section.

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Credit author statement

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Declaration of competing interest

The authors declare no conflict of interest.

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Legend to Figures

Figure 1. Quantification efficiency of RW samples at different pH in 50 mM HEPES buffer. Grey column – RW sample with unmodified PrepGEM extraction buffer. Blue columns – extraction buffer modified with 50 mM HEPES at various pH values. The quantification efficiency (%) showed the percentage of spiked *C. jejuni* quantifiable by this workflow.

Figure 2. Comparing the effectiveness of Tween 20 and Tween 80 at improving QE on RW samples. RW samples were treated with various concentrations of Tween 20 or Tween 80 prior to the capture-quantification workflow. Grey column – RW sample without Tween 20/Tween 80. Blue columns – RW samples treated with Tween 20 at 0.25–3% (v/v). Green columns – RW samples treated with Tween 80 at 1–6% (v/v). Tween 20 only – Negative control, RW sample without spiked *C. jejuni* cells, treated with 2% Tween 20. The quantification efficiency (%) showed the percentage of spiked *C. jejuni* quantifiable by this workflow.

Figure 3. Effectiveness of Tween 20 (3% v/v) at improving quantification efficiency varies with the age of the reagent. Year of manufacture (2005–2021) and age (0-16 years old) were indicated for each sample. Grey column – Untreated RW sample. Yellow columns – RW treated with older (15–16 Y) Tween 20; years of manufacture 2015 and 2016. Blue columns – RW treated with newer (0–6 Y) Tween 20 from various suppliers as detailed in the methods section. Green column – RW treated with de-esterified Tween 20 synthesised by acid-catalysed methanolysis of new (0 Y) Sigma Tween 20. The quantification efficiency (%) showed the percentage of spiked *C. jejuni* quantifiable by this workflow.

Figure 4. ^{1}H NMR spectra (400 MHz, DMSO-d_6) of five commercial Tween 20 samples ranging in age from 0 to 16 years old, compared to an authentic standard of lauric acid (LA). The percent LA composition was determined by comparing the relative integrals for the methylene triplet signals at d_H 2.27 (Tween 20) and d_H 2.17 (LA).

Figure 5. De-esterification of Tween 20 by acid-catalysed methanolysis.

Figure 6. Effect of combined treatment on the quantification efficiency compared to single methods of Tween 20 addition and the HEPES-modified extraction buffer. Stock of Tween 20 used refers to Table 1; Old T20 = T20-2005-AMRESCO; New T20 = T20-2021-Sigma. Grey columns – Untreated RW samples. Light blue columns – RW samples treated with T20 only (3% v/*v*). Dark blue columns – RW samples treated with HEPES buffer only (50 mM, pH 6.0). Navy blue columns – RW samples treated with both HEPES buffer and T20. Light green columns – direct pH adjustment with dilute hydrochloric acid. Dark green columns – combined treatment of T20 and direct pH adjustment. S1–S6 – Sample number 1 through 6, collected from the same location over a period of one year.

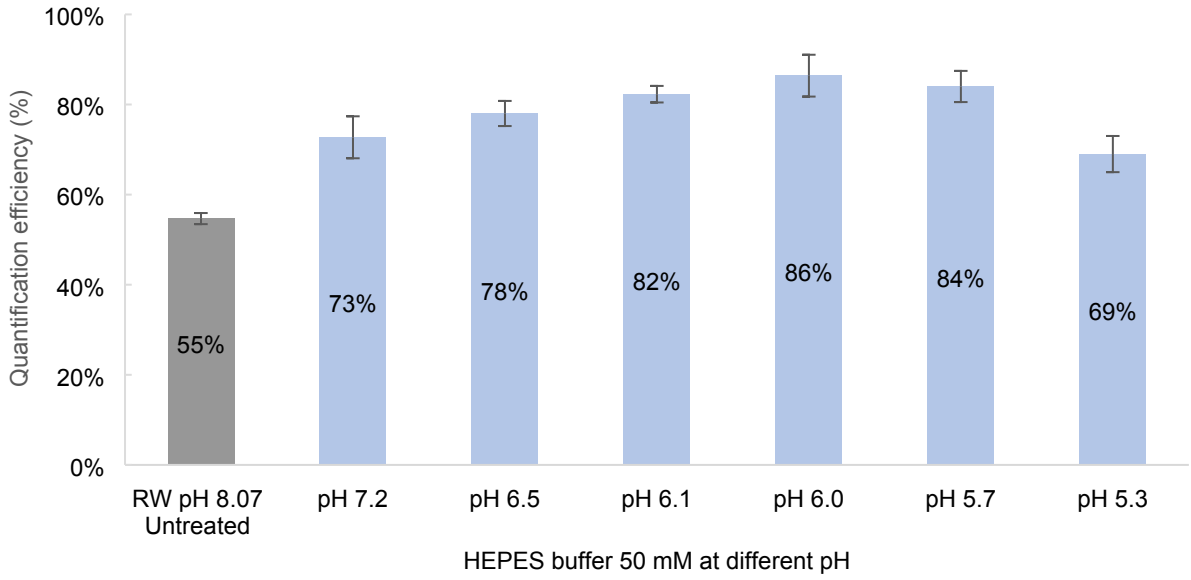
100%
80%
60%
40%
20%
0%
Quantification efficiency (%)
55%
73%
78%
82%
86%
84%
69%
RW pH 8.07 Untreated
pH 7.2
pH 6.5
pH 6.1
pH 6.0
pH 5.7
pH 5.3
HEPES buffer 50 mM at different pH

Figure 1

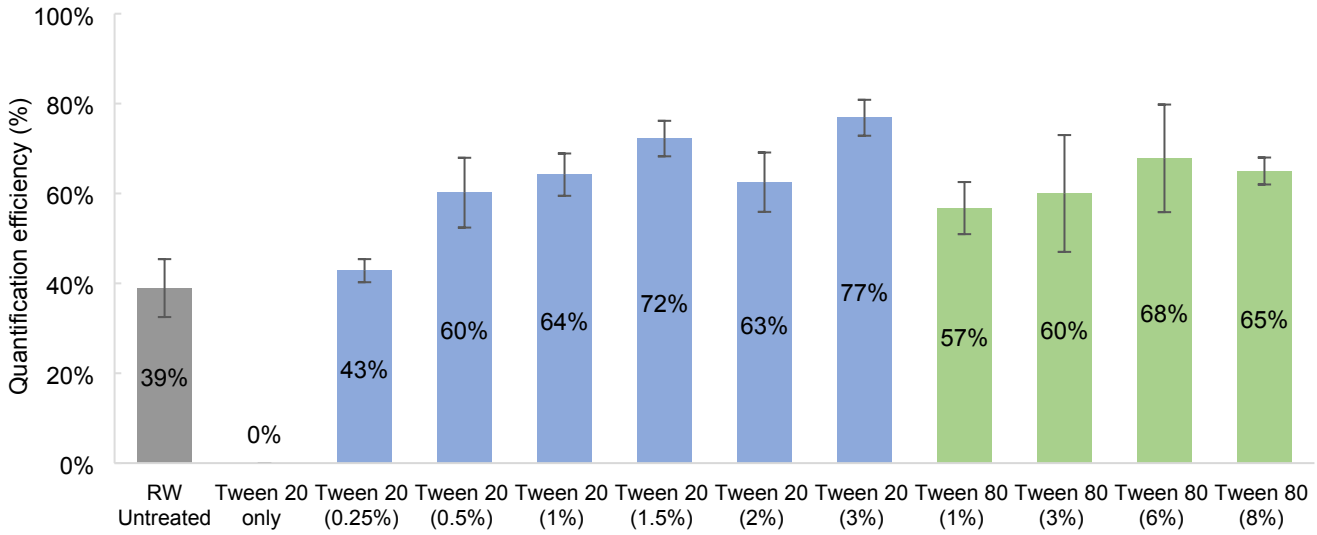
Quantification efficiency (%)
100%
80%
60%
40%
20%
0%
39%
0%
43%
60%
64%
72%
63%
77%
57%
60%
68%
65%
RW Untreated
Tween 20 only
Tween 20 (0.25%)
Tween 20 (0.5%)
Tween 20 (1%)
Tween 20 (1.5%)
Tween 20 (2%)
Tween 20 (3%)
Tween 80 (1%)
Tween 80 (3%)
Tween 80 (6%)
Tween 80 (8%)

Figure 2

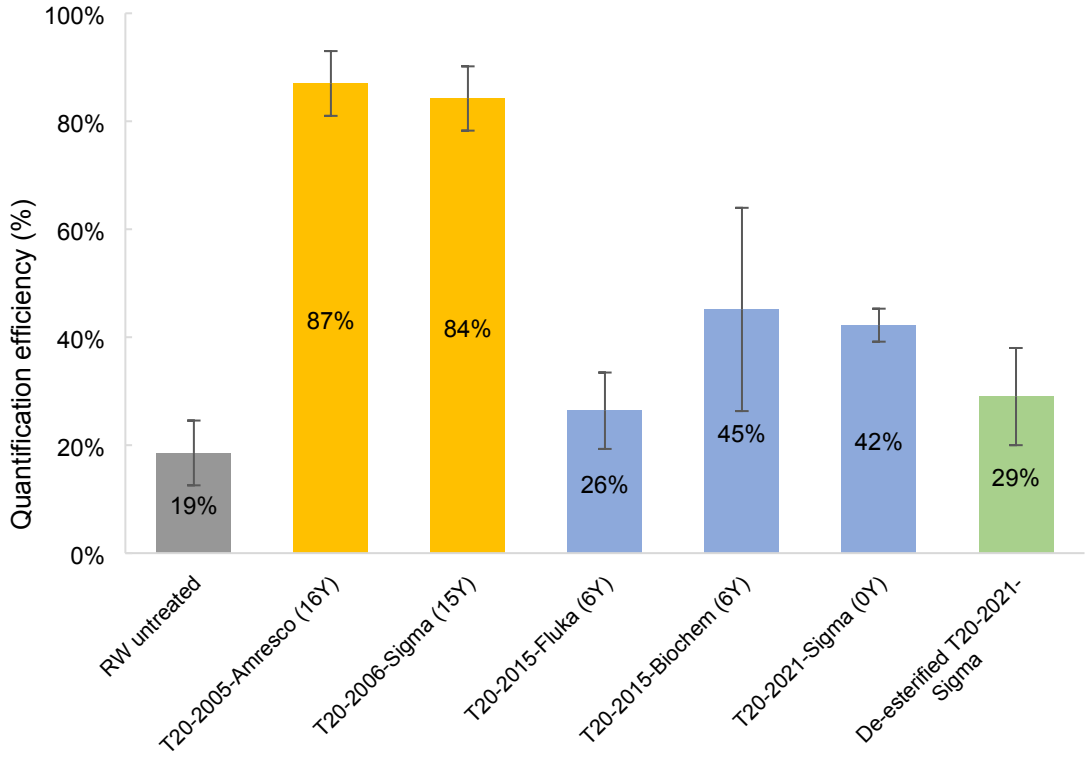
100%
80%
60%
40%
20%
0%
Quantification efficiency (%)
19%
87%
84%
26%
45%
42%
29%
RW untreated
T20-2005-Amresco (16Y)
T20-2006-Sigma (15Y)
T20-2015-Fluka (6Y)
T20-2015-Biochem (6Y)
T20-2021-Sigma (0Y)
De-esterified T20-2021-Sigma

Figure 3

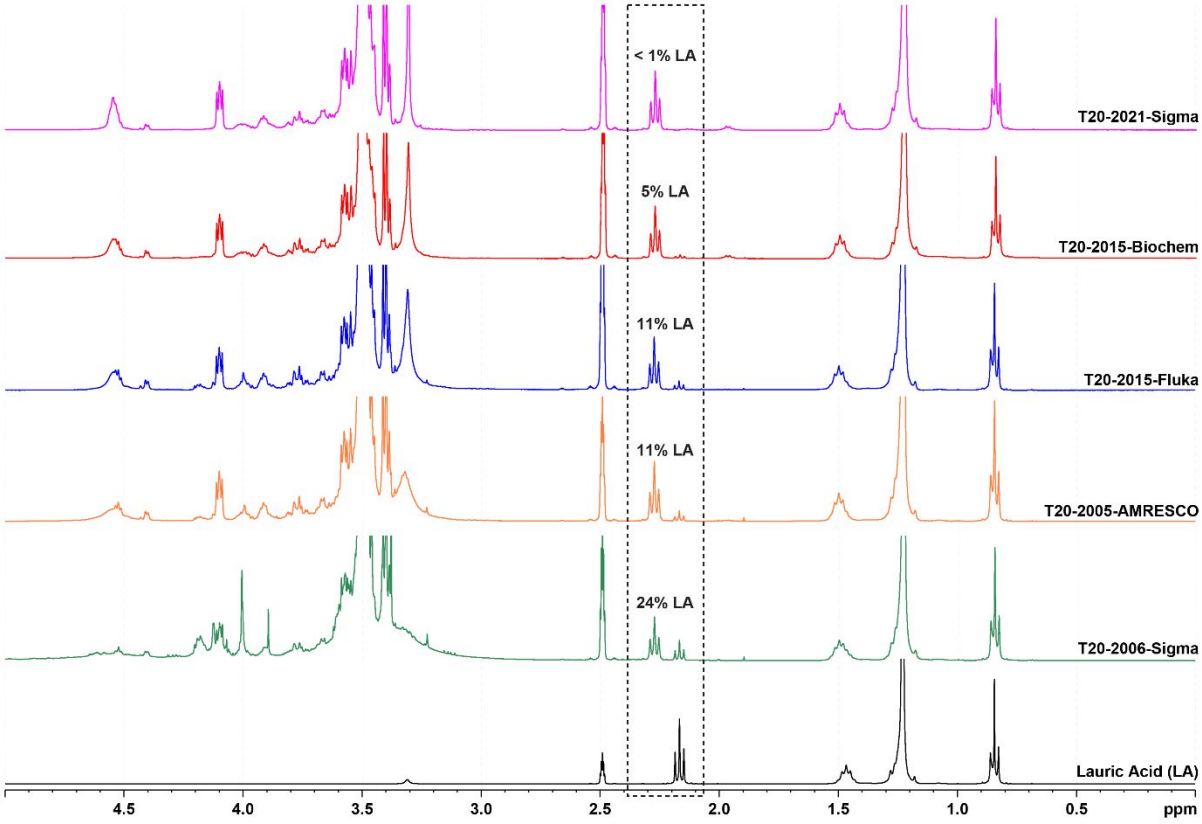
< 1% LA
T20-2021-Sigma
5% LA
T20-2015-Biochem
11% LA
T20-2015-Fluka
11% LA
T20-2005-AMRESCO
24% LA
T20-2006-Sigma
Lauric Acid (LA)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm

Figure 4

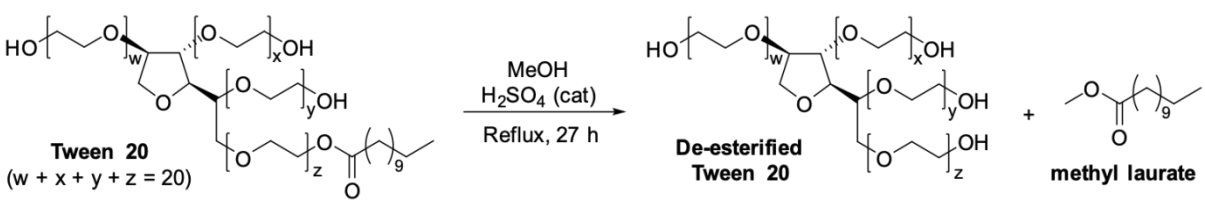
Tween 20
(w + x + y + z = 20)
MeOH
H2SO4 (cat)
Reflux, 27 h
De-esterified
Tween 20
methyl laurate

Figure 5

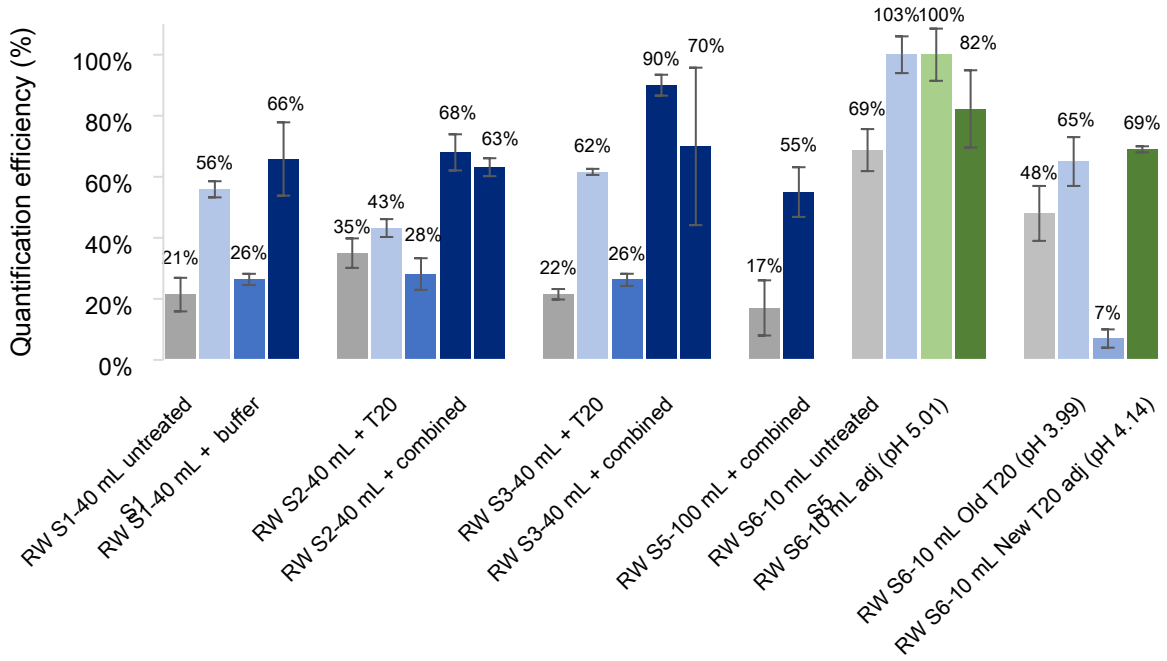
Quantification efficiency (%)
100%
80%
60%
40%
20%
0%
21%
56%
26%
66%
35%
43%
28%
68%
63%
22%
62%
26%
90%
70%
17%
55%
69%
103%
100%
82%
48%
65%
7%
69%
RW S1-40 mL untreated
RW S1-40 mL + buffer
RW S2-40 mL + T20
RW S2-40 mL + combined
RW S3-40 mL + T20
RW S3-40 mL + combined
RW S5-100 mL + combined
RW S6-10 mL untreated
RW S6-10 mL adj (pH 5.01)
RW S6-10 mL Old T20 (pH 3.99)
RW S6-10 mL New T20 adj (pH 4.14)

Figure 6

Table 1. Tween 20 and Tween 80 supplier and reagent information.

Reagent Name	Type	Catalogue Number	Reagent Grade	Manufacturer	Year of Manufacture	Hydrolysis
T20-2005-AMRESCO	T20	0777-1L	Reagent	AMRESCO	2005	11%
T20-2006-Sigma	T20	P5927-100ML	Electrophoresis	Sigma	2006	24%
T20-2015-Fluka	T20	P7949H 250ML	Electrophoresis	Fluka	2015	11%
T20-Biochem	T20	BIO0777-500ML	Biotech	Biochemicals, Astral Scientific	2015	5%
T20-2021-Sigma	T20	P7949-500ML	BioXtra	Sigma-Aldrich	2021	< 1%
T80-2015-Sigma	T80	P5188-100ML	Molecular Biology	Sigma	2015	13%

T20, Tween 20; T80, Tween 80

Table 2. Initial evaluation of treatment strategies for RW samples. Sample volume - 40 mL unless otherwise listed. Quantification efficiency (QE) represents the percentage of *C. jejuni* cells detected from specific samples compared to number of cells spiked in. SD – standard deviation.

Treatment methods	QE	SD	Comment
Untreated samples	20-67%	-	
Centrifugation	13%	6%	
Filtration post extraction	44-52%	4%	DNA filtered via 0.2 μm filter after extraction
PrepGEM enzyme (1 μL, control)	60%	1%	1 μL prepGEM enzyme per 100 μL reaction mix
Extra prepGEM enzyme (4 μL)	50%	4%	4 μL prepGEM enzyme per 100 μL reaction mix
Tris buffer pH 6.9 (20 mM)	64%	5%	Buffer added prior to DNA extraction. Sample volume 20 mL
Tris buffer pH 6.9 (50 mM)	63%	10%	
HEPES buffer pH 7.2 (50 mM)	47%	5%	
HEPES buffer pH 7.2 (100 mM)	48%	8%	
MOPS buffer pH 5.97 (50 mM)	6%	7%	Buffer added prior to DNA extraction
MOPS buffer pH 6.36 (50 mM)	7%	10%	
MOPS buffer pH 6.67 (50 mM)	10%	4%	
Sulfuric acid wash (1.25% v/v)	35%	6%	Filters washed with sulfuric acid after filtration
Sulfuric acid wash (0.5% v/v)	34%	7%	
Sulfuric acid wash (0.25% v/v)	37%	12%	
Sulfuric acid wash (0.125% v/v)	34%	1%	
NaOH wash (50 mM)	20%	1%	Filters washed with NaOH after filtration
Tween 20 (2% v/v)	63%	7%	Added to sample prior to filtration
Tween 20 (1% v/v)	63%	6%	
Tween 20 (0.5% v/v)	63%	4%	
Tween 20 (0.25% v/v)	55%	2%	

Supplementary Data

A Rapid Method for the Detection and Quantification of Pathogen *Campylobacter jejuni* from Environmental Water Samples

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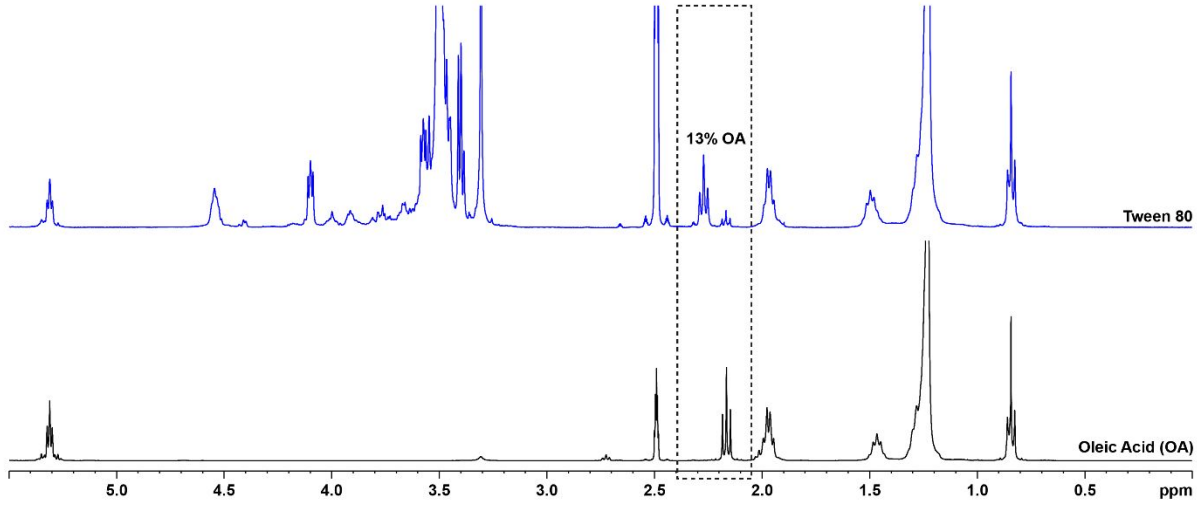
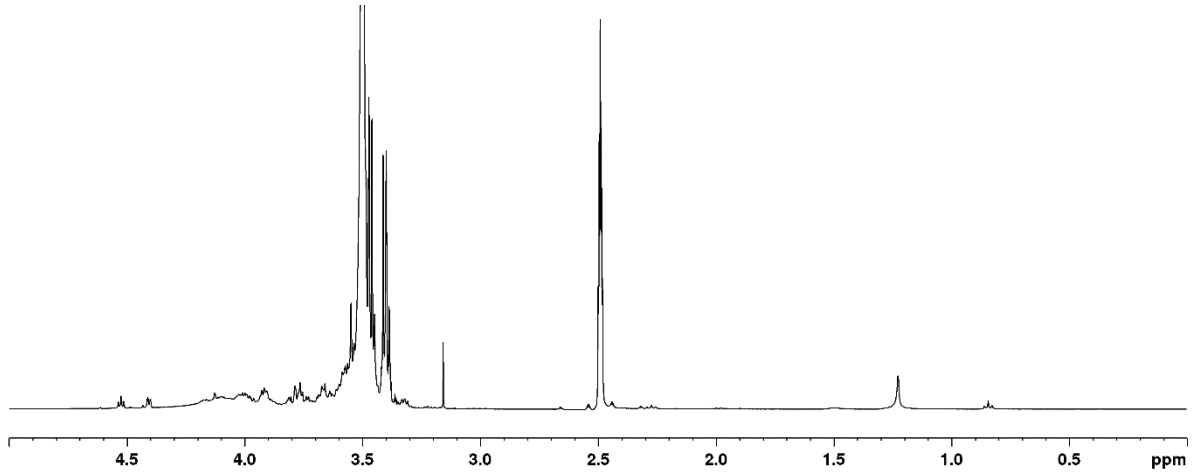
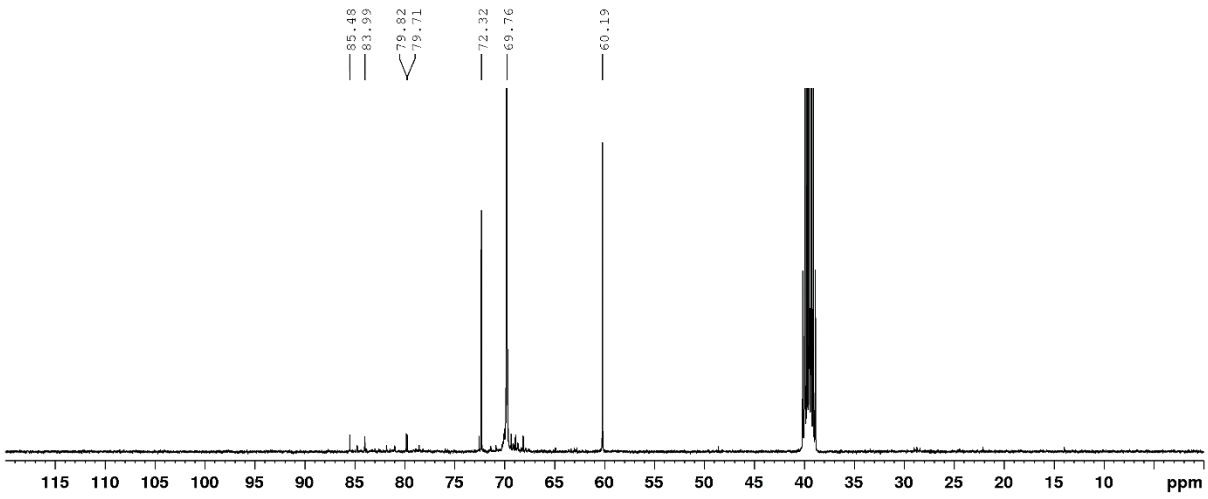


Supplementary Figure S1. ^{1}H NMR spectra (400 MHz, DMSO-d_6) of commercial Tween 80 sample (7 years old), compared to an authentic standard of oleic acid (OA). The percent OA composition was determined by comparing the relative integrals for the methylene triplet signals at d_H 2.27 (Tween 80) and d_H 2.17 (OA).



Supplementary Figure S2. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of de-esterified Tween 20.



Supplementary Figure S3. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of de-esterified Tween 20.