

Anaerobes in the microbiome

Oxygen exposure decreases the yield of high-molecular-weight DNA from some anaerobic bacteria and bacterial communities during DNA extraction

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ABSTRACT

Objectives: The central challenge in third-generation sequencing lies in meeting the requirements for DNA quality (integrity and purity) and quantity. Therefore, novel improvements in DNA extraction methods are needed to satisfy these requirements. We reasoned that in anaerobic microbial communities, the presence of certain strict anaerobes containing oxygen-activated DNase activity might contribute substantially to the poor integrity of extracted metagenomic DNA (or genomic DNA from some pure cultures) if exposed to air.

Methods: To test this hypothesis, we developed an enhanced genomic and metagenomic DNA isolation technique that we applied to a specifically chosen set of both strict and aerotolerant anaerobes, as well as to the hindgut microbiota of a herbivorous marine fish.

Results: Considering the quality (or degradation) of extracted DNA obtained under anaerobic versus aerobic conditions, we found that DNA extracted aerobically from cells of some strict anaerobes showed more degradation of high molecular weight DNA than analogous preparations under anaerobic conditions. In contrast, with the selected aerotolerant anaerobes, no discernible difference was found between the molecular sizes of DNA extracted aerobically and anaerobically. Metagenomic DNA extracted from the fish hindgut microbiota showed higher yields and better quality under anaerobic conditions compared to aerobic conditions.

Conclusion: Our study effectively demonstrates the advantages of our improved extraction protocol in anaerobic conditions. This is evident through the improved quality of extracted DNA. Such findings may be valuable for studies, especially metagenomic studies, where the quality and quantity of DNA are crucial for downstream analysis.

1. Introduction

The third generation of long-read sequencing technologies includes two prevalent platforms: single molecule real time (SMRT) sequencing and nanopore-based sequencing, represented respectively by Pacific Biosciences (PacBio) [1] and Oxford Nanopore Technologies (ONT) [2]. These methods offer numerous advantages over traditional short-read sequencing technologies, such as less need for complex assembly algorithms, and better understanding of complex genomic regions, structural variations, repetitive elements and even detection of epigenetic modifications [3]. However, the core challenge of third-generation sequencing is the physical integrity of extracted DNA, as large

fragments are required to span long genomic regions ranging from thousands to tens of thousands of base pairs [4]. Therefore, novel improvements in methods are needed to meet the requirements for DNA quality, including both integrity (high-molecular-weight DNA) and purity (lack of contaminants).

The critical step in the extraction of bacterial DNA is cell lysis, which involves either chemical (detergent) or a combination of chemical and physical (bead beating) treatments [5,6]. During this step, the genomic or metagenomic DNA is highly exposed to several factors but the effect of oxygen on DNA degradation (particularly in certain types of bacteria or mixtures of bacteria from anaerobic environments) is often overlooked. Oxygen can impact the process of DNA extraction in several

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ways, primarily by promoting the activity of enzymes that degrade DNA, known as deoxyribonucleases or DNases [7]. Some of these enzymes can degrade the extracted DNA, even in the presence of strong inactivating enzyme agents such as denaturing detergents and/or chelating chemicals, leading to lower yields of high-molecular-weight DNA and compromised quality of DNA [8]. It is well known that various anaerobic bacteria are substantial sources of deoxyribonuclease activity [9,10].

We reasoned that in anaerobic microbial communities, the presence of certain strict anaerobes containing oxygen-activated DNase activity might contribute substantially to the poor quality and quantity of extracted metagenomic DNA (or genomic DNA from some pure cultures) if exposed to air during DNA isolation prior to the denaturation of oxygen-activated DNase activity.

To test this hypothesis, we developed an enhanced genomic and metagenomic DNA isolation protocol that we applied to a specifically chosen set of both strict and aerotolerant anaerobes, as well as to the hindgut microbiota of the herbivorous marine fish *Kyphosus sydneyanus*. Our aim was to compare the quality (or degradation) of extracted DNA under anaerobic versus aerobic conditions. The results of these experiments not only facilitated the identification of necessary modifications, but also led to the refinement of a more effective method for extraction of metagenomic and genomic DNA from strict anaerobes. Our work revealed that conducting the initial extraction steps under a strictly anaerobic environment effectively reduces the risk of DNA degradation. This outcome holds true not only for pure bacterial strains but also for some anaerobic communities.

2. Methods

2.1. Preparation of pure bacterial strains for genomic DNA extraction

High-molecular-weight genomic DNA was extracted from three bacterial strains: *Alistipes* BP38, *Prevotella* strain RS2, and *Lactobacillus gasseri* ATCC 9857. These strains were specially selected for comparative purposes, representing strict anaerobes (*Alistipes* BP38 and *Prevotella* strain RS2) and aerotolerant anaerobes (*Lactobacillus gasseri* ATCC 9857). The culture isolation, media, and conditions for all three strains are as follows:

Alistipes strain BP38 was isolated from section IV of the hindgut of *K. sydneyanus*. Six adult individual fishes were collected by underwater spear near Great Barrier Island in the Hauraki Gulf, New Zealand (36°0.165'S; 175.301'E) in 2018. Fish collection was conducted under University of Auckland Animal Ethics approval AEC-001949. The contents of the hindgut were processed aboard the University of Auckland research vessel RV Hawere as follows. The gut was removed and divided into five segments, numbered I-V, as described previously [11]. The contents of section IV of the hindgut of a freshly captured *K. sydneyanus* were transported to the laboratory under anaerobic conditions. The tube of gut contents was placed inside a vinyl anaerobic chamber (Coy Laboratory Products) maintained with N₂:H₂:CO₂ (87.5:7.5:5) (H₂ levels at 3% inside the chamber). Solids were allowed to settle and then 100 µL of the supernatant fluid was plated on modified YCFA (yeast extract-casein hydrolysate-volatile fatty acids) [12] agar plates (with modifications as described in Supplementary Note 1) and incubated in an anaerobic jar under an atmosphere of 47.5% (v/v) N₂, 47.5% CO₂ and 5% H₂. Colonies appeared after five days of incubation at 19 °C. One of the colonies was isolated as a pure culture by successive re-streaking and designated isolate BP38. An *Alistipes* BP38 was stored as a 25% glycerol suspension and separately as a stab in agar medium at -80 °C. The identification of isolated pure colonies was performed by colony PCR. The 16S rRNA gene was amplified using the primer set of the forward primer F_0008 (5'-AGAGTTTGATYMTGGCTCAG-3') and the reverse primer R_1510.

(5'-ACGGYTACCTTGTTACGACTT-3'). The entire length of the 16S rRNA gene was sequenced in two directions using Sanger sequencing (ABI PRISM 3130XL Genetic Analyzer, Applied Biosystems). For

identification purposes, the Basic Local Alignment Search Tool (BLAST) was used to compare the full-length 16S rRNA gene sequence against SILVA 16S rRNA gene database.

Prevotella strain RS2 was originally isolated on mucin from pig colonic mucosa as substrate [13]. It was grown for the present research work anaerobically at 37 °C under CO₂ in liquid medium as described in Ref. [14] with the following modifications. The energy source was 0.02% (w/v) galactose. In addition, trace elements solution 0.01% (v/v) and volatile fatty acid mixture 0.31% (v/v) were added to the medium as described [15]. For preparation of solid medium plates used for bacterial cell growth, 1.7% Agar (Difco) was added to the liquid medium.

The aerotolerant *Lactobacillus gasseri* ATCC 9857 strain was grown aerobically at 37 °C on MRS agar and broth (Difco).

2.2. Genomic DNA extractions from pure bacterial strains under aerobic and anaerobic conditions

Genomic DNA was extracted from cells of two strict anaerobes (*Alistipes* BP38 and *Prevotella* RS2) and one aerotolerant anaerobe (*Lactobacillus gasseri* ATCC 9857) under aerobic and anaerobic conditions following (Sambrook & Russell [16] with modifications in the first three steps - harvesting, washing and cell lysis. The initial step of cell harvesting was performed anaerobically inside a Coy anaerobic chamber in an atmosphere as described above. The cells were harvested from plates by adding anaerobic YCFA-M liquid medium using a spreader and a pipette as described above and transferring the cell suspension into two 2 mL microcentrifuge tubes, each with a density of approximately 3×10^9 cells per mL. The first tube, labelled "plus oxygen", was removed from the chamber, while the second, labelled "minus oxygen", was retained inside the chamber. The next steps for the "plus oxygen" and "minus oxygen" samples were carried out aerobically and anaerobically, respectively, as follows. Cells were centrifuged at 5000×g for 5 min and resuspended in 1000 µL Lysozyme Lysis Buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA pH 8.0, 1% TritonX-100). Lysozyme was added to a final concentration of 2 mg/mL and the cell suspension was incubated for 30 min at 37 °C. The final concentrations of 2% SDS and 2.5 mg/mL Proteinase K were added and the cells were incubated for 40 min at 56 °C. The remaining steps of protein removal with phenol-chloroform, RNase treatment and DNA precipitation were conducted under aerobic conditions for both samples following Sambrook & Russell [16].

In addition, *Alistipes* BP38 strain, unlike *Prevotella* and *Lactobacillus*, produces a significant amount of polysaccharide, which requires an additional step in the extraction process. This step is adapted from the "CTAB modified method" as described in Wilson [17]. Briefly, after anaerobic incubation of the cells with SDS and proteinase K for 40 min at 56 °C, a stock solution of 5M NaCl was added to the final concentration of 0.7 M. Then, 0.1 volume of CTAB/NaCl solution was transferred into the mixture and it was incubated at 65 °C for 10 min. An equal volume of chloroform/isoamyl alcohol was added and the tube centrifuged at 20,000×g for 10 min. The upper aqueous phase was transferred to a new tube and the proteins removed by using the steps described above for phenol-chloroform extraction, RNase treatment and DNA precipitation [16].

2.3. Metagenomic DNA extraction from hindgut samples of *K. sydneyanus* under aerobic and anaerobic conditions

Metagenomic DNA was extracted from the entire hindgut content of gut section V, obtained from four *K. sydneyanus* individuals (G201, G202, G204 and G205), collected as described in Pardesi et al. [18] and from three individuals (G242, G246 and G248) collected as described in Pisaniello et al. [19]. The fishes were promptly removed from the water and processed on board the University of Auckland research vessel RV Hawere. The gut content samples from each fish were collected in 15 mL Falcon tubes and immediately frozen in liquid nitrogen. Subsequently, the samples were stored at -80 °C in the laboratory until processing. We

followed the DNA extraction protocol outlined by Pardesi et al. [18] with modifications applied to the initial step that involved mechanical disruption of the samples. The handling of samples in the presence and absence of oxygen was as follows. Briefly, approximately 2 mL of each frozen sample was transferred from the 50 mL Falcon collection tube onto an ice-cold 30 mm Petri dish using the sharp end of a sterile inoculating loop inside the Coy anaerobic chamber. After that, 2 mL of DNA/RNA Shield (Zymo Research) was added to the sample and homogenized using a 1 mL syringe. (DNA/RNA shield is added to biological preparations to preserve the genetic integrity of samples and completely inactivates infectious agents).

The resultant mixture was transferred into two bead-beating tubes from the DNeasy PowerSoil Pro Kit (Qiagen). One tube, labelled “minus oxygen”, was placed on Genie Vortex for 5 min at max speed inside the Coy anaerobic chamber. The second tube, labelled “plus oxygen”, was removed from the chamber and placed on Genie Vortex for the same time and speed on the lab benchtop upon exposure to air. The next steps for the “plus oxygen” and “minus oxygen” samples were carried out aerobically and anaerobically, respectively, as follows. After completing the mechanical disruption step, the samples were centrifuged at 10,000×g for 1 min. The resulting supernatants were transferred to new 2 mL tubes, and solution CD2 (which utilizes patented Inhibitor Removal Technology, that effectively precipitates non-DNA organic and inorganic materials including humic substances, cell debris, and proteins) was added to these tubes to lyse the cells and denature proteins. The remaining steps were performed following the manufacturer’s instructions of the DNeasy PowerSoil Pro Kit (Qiagen). After the last step, the eluted DNA in TE buffer of the minus oxygen sample was taken out of the chamber and together with the eluted DNA of the plus oxygen sample were treated with 2 µL of RNase A/T1 mixture (2 mg/mL of RNase A and 5000 U/mL of RNase T1) and incubated at 37°C for 20 min. Removal of RNase A/T1 proteins and DNA precipitation was performed according to standard molecular techniques [16].

2.4. Measuring DNA yield, quality and integrity

DNA yield and purity were measured spectrophotometrically using the NanoDrop spectrophotometer (Thermo Fisher Scientific) as outlined in the legend of Table 1. The concentration of double-stranded DNA was quantified fluorometrically using the Qubit Fluorometer (Thermo Fisher Scientific). DNA integrity was assessed via conventional 1 % agarose gel electrophoresis or pulse field gel electrophoresis utilizing the Bio-Rad system.

Pulsed Field Gel Electrophoresis (PFGE) was performed with a CHEF Mapper XA Bio-Rad 170-3670 system using a Standard Casting Stand with a 14 × 13 cm frame and 15 well comb. The samples and markers were loaded into a 1 % pulsed field certified agarose (Bio-Rad) gel in 0.5 x TBE running buffer at 14 °C for 18 h. The selected parameters for the CHEF system were as follows: voltage gradient of 6 V/cm; pulse angle of

120 °C and the initial and final pulses conducted for 1.19–26.29 s, respectively. After electrophoresis, the gels were stained with ethidium bromide (5 µg/mL) and imaged according to the manufacturer’s user guide. DNA markers used are a 1 Kb plus DNA ladder (Invitrogen) and a high-molecular-weight CHEF DNA standard (Biocompare).

Densitometric image analysis was performed using an iBright FL1500 Imaging System (Thermo Fisher Scientific) with iBright Analysis Software. The high molecular weight DNA fraction (fragments above 10 kb) was quantified densitometrically and expressed as a percentage of the total DNA and defined as relative densitometric high-molecular weight values (RD_{HMWV}). This measurement was normalized based on the yield from each sample run on a 1 % agarose gel electrophoresis. Images were analysed in manual mode for relative quantitation.

2.5. Statistical analysis

Statistical analysis was performed using a student’s t-test to compare DNA quantity and quality in the presence and absence of oxygen. This analysis was applied to genomic DNA extractions from the three bacterial strains *Alistipes* BP38, *Prevotella* strain RS2, and *Lactobacillus gasseri* ATCC 9857, combinations of pairs of these species, and metagenomic DNA extractions from five fish (G201, G202, G242, G246, and G248). A p-value of ≤0.01 was considered statistically significant.

3. Results

3.1. Aerobic and anaerobic extraction of DNA from bacterial cells

To compare DNA extraction under aerobic and anaerobic conditions, we intentionally chose two strict anaerobic strains, *Alistipes* BP38 and *Prevotella* RS2, along with one aerotolerant anaerobic strain, *Lactobacillus gasseri* ATCC 9857. Here, we hypothesize that one or both of the selected strict anaerobic strains may contain oxygen-activated DNases, which would degrade DNA extracted under aerobic conditions. In contrast, we do not anticipate a significant difference in DNA degradation of extracted genomic DNA under aerobic and anaerobic conditions for the aerotolerant anaerobic strain.

In order to determine whether air (oxygen) altered the measured DNA yield and purity, we performed DNA extractions from cells of *Prevotella* strain RS2, *Alistipes* BP38 and *Lactobacillus gasseri* ATCC 9857, on each of these species alone and also combinations of pairs of these species mixed together, both under aerobic and anaerobic conditions. The results of these experiments are detailed in Table 1. The first row in this Table shows the results with *Prevotella* alone. The yield of DNA after extraction showed an improved yield anaerobically compared to aerobically of 38.9 % ± 2.8 % (measured fluorometrically) and 47.9 % ± 3.2 % (measured spectrophotometrically). The corresponding yields of DNA for *Alistipes* (second row) were improved anaerobically by 22.1 % ± 2.9 % (measured fluorometrically) and 16.3 % ± 1.8 % (measured

Table 1
Quantity and quality of DNA extracted from anaerobic bacterial species and their mixtures in the presence (+ O₂) and absence (-O₂) of oxygen. DNA yield was measured by (i) fluorometric and (ii) spectrophotometric measurements. DNA purity was measured by (iii) A260nm/A280nm and (iv) A260nm/A230nm ratios, as explained in the text. Values are expressed as means with standard deviations (SD) (n = 3).

Sample	Yield ^{ng} /µl Mean (SD)		Yield ^{fl} ng/µl Mean (SD)		A260nm/A280nm Mean (SD)		A260nm/A230nm Mean (SD)	
	-O ₂	+O ₂	-O ₂	+O ₂	-O ₂	+O ₂	-O ₂	+O ₂
<i>Prevotella</i> RS2	96.4 (±4.01)	69.3 (±4.04)	319.0 (±10.15)	215.7 (±12.74)	1.84 (±0.05)	1.82 (±0.07)	2.19 (±0.04)	2.21 (±0.08)
<i>Alistipes</i> BP38	57.0 (±4.58)	46.7 (±4.73)	244.3 (±14.01)	210.0 (±19.31)	1.75 (±0.08)	1.70 (±0.11)	1.91 (±0.09)	1.86 (±0.12)
<i>L. gasseri</i> 9857	124.0 (±4.36)	107.3 (±8.33)	692.3 (±10.79)	713.0 (±15.13)	1.87 (±0.05)	1.93 (±0.06)	2.09 (±0.03)	2.23 (±0.03)
<i>Prevotella</i> RS2 + <i>Alistipes</i> BP38	57.6 (±4.04)	42.5 (±4.92)	262.3 (±10.79)	208.7 (±17.62)	1.83 (±0.09)	1.78 (±0.09)	2.02 (±0.10)	1.93 (±0.14)
<i>Prevotella</i> RS2 + <i>L. gasseri</i> 9857	85.0 (±4.58)	69.3 (±4.04)	309.7 (±14.98)	253.0 (±12.77)	1.89 (±0.04)	1.94 (±0.03)	2.06 (±0.07)	2.12 (±0.08)
<i>Alistipes</i> BP38 + <i>L. gasseri</i> 9857	81.3 (±3.51)	72.7 (±6.03)	361.7 (±15.53)	288.3 (±15.95)	1.76 (±0.12)	1.80 (±0.12)	1.90 (±0.10)	1.86 (±0.11)

spectrophotometrically). The third row shows that the DNA yield for the aerotolerant *Lactobacillus* bacterium increased anaerobically by $15.5\% \pm 1.3\%$ (measured fluorometrically) while there was a decrease anaerobically by $3\% \pm 0.1\%$ (measured spectrophotometrically) respectively, indicating that for the aerotolerant bacterium example anaerobic extraction was only marginally better, if at all. The same trends of better DNA yields were also observed when the DNA from mixtures of pairs of bacterial types were extracted together: *Prevotella* + *Alistipes* showed an increase in DNA extraction ($35.7\% \pm 4.8\%$ and $25.7\% \pm 2.4\%$, row 4), *Prevotella* + *Lactobacillus* showed an increase ($22.6\% \pm 1.8\%$ and $22.4\% \pm 1.6\%$, row 5), and *Alistipes* + *Lactobacillus* also showed an increase ($12.0\% \pm 1.1\%$ and $25.4\% \pm 1.8\%$, row 6). The spectrophotometric method measures the double bonds in the N-rings of all nucleotides (double-stranded and single-stranded DNA and RNA), whereas the fluorometric method measures just the nucleotides within double-stranded DNA [20]. The *t*-test showed a *p*-value of 0.001 for DNA yields measured fluorometrically and a *p*-value of 0.01 for DNA yields measured spectrophotometrically, indicating that the differences in DNA yields between the minus oxygen (anaerobic) and plus oxygen (aerobic) conditions were statistically significant.

The purity of the extracted DNA was assessed by measuring two ratios: A260/280 and A260/230. These ratios help to identify different types of contamination. The A260/280 ratio assesses protein and RNA contamination, with the recommended optimal range between 1.8 and 2.0 [20]. Our analysis showed that the A260/280 ratios for all extracted DNA samples ranged from 1.7 to 1.94, indicating minimal protein contamination (see Table 1, third column). On the other hand, the A260/230 ratio is used to assess the presence of organic compounds such as chaotropic salts, phenol or benzene derivatives, which can be part of humic acids [21]. The optimal range for A260/230 is between 1.8 and 2.2. Our results showed that all A260/230 ratios were within this range, indicating the absence of organic contaminants (see Table 1, fourth column).

3.2. Pulse field electrophoresis of purified DNA extracted from cells in the presence and absence of oxygen using the three bacterial species and their mixtures

Pulse field electrophoresis can be used to show how degraded high-molecular-weight DNA from cells has become during extraction (Fig. 1). The two standards show 5–15 kb bands (lane 7 and 15) and 8.3–129.9 kb bands (lane 8). Two distinct batches of DNA extracted from *Prevotella* cells are illustrated in lanes 1–4. More precisely, lanes 1 and 3 represent DNA extracted from *Prevotella* under aerobic conditions, while lanes 2 and 4 show analogous DNA preparations from *Prevotella* cells extracted under anaerobic conditions. The aerobic extraction yielded DNA of a much lower molecular size, suggesting that oxygen-activated DNase activity was occurring during the aerobic extraction. Lanes 5 and 6 show DNA extracted aerobically and anaerobically, respectively, from a mixture of *Prevotella* and *Lactobacillus* cells. Lane 5 showed more degradation of high-molecular-weight DNA. The control DNA extraction of *Lactobacillus* alone is shown in lanes 9 (aerobic) and 10 (anaerobic). These indicate no discernible difference between the DNA molecular sizes. Lanes 11 and 12 show DNA extracted aerobically and anaerobically, respectively, from a mixture of *Alistipes* and *Lactobacillus* cells, showing DNA was more degraded in the aerobic extractions. Similarly, the control extractions of *Alistipes* alone (lanes 13 and 14) also showed more DNA degradation aerobically. Thus overall, the *Alistipes* and *Prevotella* DNA samples extracted aerobically from cells showed signs of oxygen-activated DNase activity, whilst the aerotolerant *Lactobacillus* DNA that was extracted aerobically did not. Whenever *Prevotella* or *Alistipes* cells mixed with *Lactobacillus* cells were extracted, the ensuing mixture of DNA showed degradation when the extraction was done aerobically.

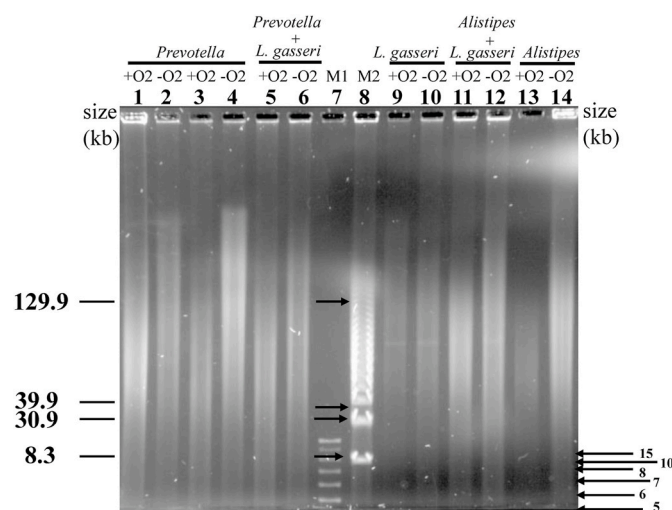


Fig. 1. Pulsed Field Gel Electrophoresis of genomic DNA extracted from bacterial species and their mixtures in the presence of air (+ O₂) and anaerobically (-O₂). The genomic DNA was extracted as follows from: Lanes 1–4: a *Prevotella* strain RS2 (biological replicates) in the presence of oxygen (lanes 1 and 3) and absence of oxygen (lanes 2 and 4). Lanes 5 and 6: a mixture of *Prevotella* strain RS2 and *L. gasseri* 9857 in the presence and absence of oxygen, respectively. Lanes 7 and 8: Markers. Lanes 9 and 10: a *L. gasseri* 9857 strain in the presence and absence of oxygen, respectively. Lanes 11 and 12: a mixture of *Alistipes* BP38 and *L. gasseri* 9857 in the presence and absence of oxygen, respectively. Lanes 13 and 14: a *Alistipes* BP38 strain in the presence and absence of oxygen, respectively. Genomic DNA was electrophoresed on a 1% agarose gel, stained with EtBr and photographed in a Bio-Rad gel imaging system.

3.3. Degradation of isolated metagenomic DNA from fish gut of *Kyphosus sydneyanus* under anaerobic and aerobic conditions

Metagenomic DNA was isolated from the gut section V of seven *K. sydneyanus* individuals. The results presented in Table 2 indicate that DNA yields measured fluorometrically were higher under anaerobic conditions compared to aerobic conditions. Both the A260/280 and A260/A230 ratios in Table 2 were within acceptable ranges, suggesting that the DNA was relatively pure without substantial protein and organic contaminants. The *t*-test showed a *p*-value of 0.003 for DNA yields measured fluorometrically and a *p*-value of 0.009 for DNA yields measured spectrophotometrically, indicating that the differences in DNA yields between the minus oxygen (anaerobic) and plus oxygen (aerobic) conditions are statistically significant.

Fig. 2 illustrates the DNA fragmentation pattern of five fish samples analysed by agarose gel electrophoresis. The metagenomic DNA extracted under anaerobic conditions using our improved protocol clearly exhibited mainly high molecular-weight DNA and few short fragments. In contrast, DNA extracted under aerobic conditions displayed a pattern characterized by few high molecular-weight bands and an abundance of shorter fragments. For comparison in lanes 13 and 14 two samples G242 and G246 are shown in which DNA was extracted using the standard DNeasy PowerSoil Pro Kit aerobically, resulting in more degraded DNA with even less high-molecular bands and more short fragments.

To quantify the DNA fragmentation with and without oxygen, we used densitometric image analysis as described in the methods section. The analysis showed differences ranging from a substantial six-fold in sample G202 to a two-fold in sample G248 (see Table 3). A *t*-test was performed to determine if the effect of oxygen on DNA fragmentation was statistically significant or due to random chance. The *t*-value of 4.2 and *p*-value <0.003 indicate that the observed differences are statistically significant. DNA from fish gut samples G204 and G205 was not included in the *t*-test analysis, but the DNA fragmentation patterns were

Table 2

Quantity and quality of metagenomic DNA extracted from the hindgut of *K. sydneyanus* seven fishes in the absence (-O₂) and presence (+O₂) of oxygen. Measurements were taken using fluorometric (i) and spectrophotometric (ii) methods. Values are expressed as means with standard deviations (SD) (n = 3).

Sample	Yield ⁱ ng/μl		Yield ⁱⁱ ng/μl		A260nm/A280nm		A260nm/A230nm	
	Mean (SD)		Mean (SD)		Mean (SD)		Mean (SD)	
	-O ₂	+O ₂	-O ₂	+O ₂	-O ₂	+O ₂	-O ₂	+O ₂
G201	31.6 (±4.1)	21.7 (±3.1)	39.3 (±4.8)	34.2 (±2.4)	1.6 (±0.11)	1.7 (±0.02)	1.6 (±0.16)	1.7 (±0.04)
G202	97.2 (±4.2)	48.1 (±5.5)	107.6 (±5.8)	54.2 (±2.3)	1.8 (±0.02)	1.7 (±0.03)	1.7 (±0.06)	1.8 (±0.04)
G204	44.9 (±5.7)	24.0 (±2.6)	163.9 (±4.1)	126.0 (±4.8)	1.6 (±0.15)	1.7 (±0.06)	1.8 (±0.10)	1.8 (±0.15)
G205	61.8 (±3.6)	32.0 (±3.1)	155.6 (±5.7)	119.0 (±3.6)	1.9 (±0.06)	1.7 (±0.04)	1.7 (±0.07)	1.8 (±0.09)
G242	69.3 (±3.2)	15.4 (±3.1)	78.78 (±4.6)	20.1 (±3.3)	1.7 (±0.04)	1.6 (±0.04)	1.7 (±0.09)	1.6 (±0.07)
G246	105.3 (±3.1)	57.2 (±3.7)	115.0 (±3.5)	124.0 (±5.0)	1.8 (±0.01)	1.7 (±0.05)	1.7 (±0.08)	1.8 (±0.08)
G248	105.3 (±3.4)	14.0 (±2.8)	100.5 (±4.2)	23.9 (±4.7)	1.8 (±0.02)	1.7 (±0.06)	1.8 (±0.01)	1.7 (±0.03)

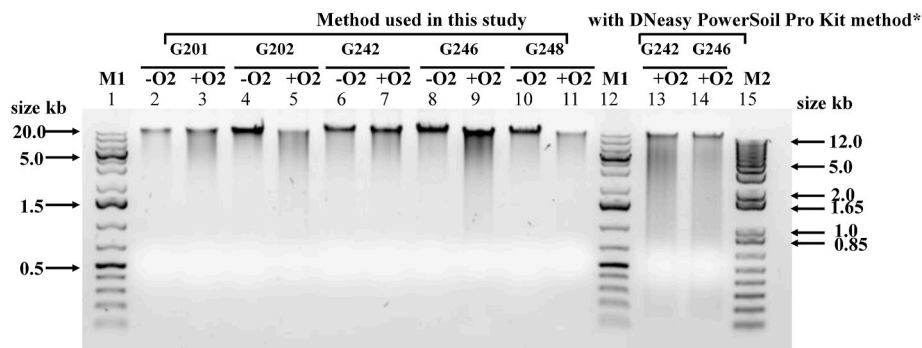


Fig. 2. Agarose (1 %) Gel Electrophoresis of metagenomic DNA isolated from *K. sydneyanus* five fish gut samples in the presence (+O₂) and absence (-O₂) of oxygen. Metagenomic DNA isolated from G201, G202, G242, G246, and G248 fish gut samples in the absence (-O₂), lanes: 2, 4, 6, 8, 10 and presence (+O₂) of oxygen, lanes: 3, 5, 7, 9, and 11, using the method described in this study. Lanes: 13 and 14, metagenomic DNA extracted from fish gut samples G242 and G246 in the presence (+O₂) of oxygen, using the standard DNeasy PowerSoil Pro Kit. Lanes 1 and 12: GeneRuler 1 kb Plus DNA Ladder (M1). Lane 15: 1 kb Plus DNA Ladder Invitrogen (M2).

Table 3

Relative Densitometric High-Molecular-Weight Values of DNA extracted from five fish gut samples under anaerobic (-O₂) and aerobic (+O₂) conditions. *RD_{HMW}V: Relative Densitometric High Molecular Weight Values are expressed in arbitrary units, calculated as described in the Methods section. **Fold Difference: The ratio of RD_{HMW}V in anaerobic conditions to aerobic conditions.

Sample	RD _{HMW} V*		Fold difference**
	-O ₂	+O ₂	
G201	66.1 (±3.5)	17.7 (±0.9)	3.7
G202	49.3 (±1.3)	8 (±1.9)	6.1
G242	39.6 (±3.6)	27.6 (±2.6)	1.4
G246	44.1 (±4.3)	10.1 (±1.1)	4.5
G248	27.6 (±2.3)	11.6 (±1.0)	2.4

consistent with the other samples (see Supplementary Fig. 1).

Overall, these results clearly show that better quality DNA was obtained anaerobically than aerobically in all extractions of seven fishes.

4. Discussion

We have shown that both the quality and quantity of extracted DNA from anaerobic bacteria and from an anaerobic gut microbiome containing a huge variety of bacterial cells is determined by the presence or absence of oxygen. The benefits of our improved genomic and metagenomic DNA extraction protocols under anaerobic conditions give higher DNA yields and better DNA quality.

In this study, strain selection was determined by two main parameters. Firstly, strains were selected based on their ability to grow under either strictly anaerobic conditions (strict anaerobes) or under both anaerobic and aerobic conditions (aerotolerant anaerobes). Secondly, strains were chosen for their diverse taxonomic affiliations. The 16S

rRNA gene of the *Alistipes* BP38 strain showed its close relationship to organisms from the genus *Alistipes* in the family *Rikenellaceae*, in the phylum *Bacteroidota*. This genus was found to dominate in section V of *K. sydneyanus* hindgut microbiota [18]. The *Prevotella* strain RS2 is from the family *Prevotellaceae*, in the phylum *Bacteroidota*. These two taxonomies are in contrast to the aerotolerant bacterium *Lactobacillus gasseri* ATCC 9857, which originates from the family *Lactobacillaceae* and is classified under the phylum *Bacillota*. It is well known that the most abundant phyla in the human gut are *Bacteroidota* and *Bacillota* [22]. There is an urgent need for sequencing more complete genomes from emerging genera such as *Alistipes* and *Prevotella*, given their potential implications for human health [23,24].

Our experiments showed that DNase-producing *Prevotella* strain RS2 and *Alistipes* BP38 exhibit higher levels of DNA degradation when exposed to air, as opposed to anaerobic conditions. This difference could be attributed to the presence of DNases that are activated in aerobic environments, and that subsequently degrade cellular DNA in the presence of oxygen. Alternatively, this phenomenon could be due to DNA chain breakage under aerobic conditions by other enzymes, which normally have other functions.

We also demonstrated that the quality and yields of metagenomic DNA extracted from the gut section V of seven *Kyphosus sydneyanus* herbivorous fishes were much higher under anaerobic conditions compared to aerobic conditions. Our findings also suggested that the addition of DNA/RNA Shield to certain anaerobic microbial environments is insufficient to prevent some oxygen-activated damage to DNA (possibly caused by an oxygen activated DNase), and thus extraction of DNA under anaerobic rather than aerobic conditions yield more intact DNA. DNA/RNA shield is a propriety cocktail mixture owned by Zymo Research, which is widely recognized as the preferred method for preserving nucleic acids in biological samples compared to other methods [25,26]. However, its exact composition remains undisclosed due to

proprietary reasons. It is highly probable that the DNA/RNA shield is a combination of nuclease activity inhibitors and chemicals with antioxidant properties. These components are likely to work together to quickly inactivate nucleases and potentially prevent the formation of oxygen radicals, thus inhibiting degradation and maintaining the integrity of DNA or RNA. However, our results clearly showed that despite the presence of the DNA/RNA shield, metagenomic DNA was degraded, when the anaerobic microbial community from the fish hindgut was exposed to oxygen during DNA extraction.

Therefore, our work underscores the importance of the absence of oxygen throughout various stages of processing including sampling, storage and the initial steps of our improved extraction protocol.

It is known that in addition to oxygen-activated DNases, the presence of oxygen can trigger other factors that contribute to metagenomic DNA degradation. A notable example is the process of bead beating during mechanical disruption. This mechanical treatment has the potential to induce oxidative damage to the overall DNA structure [27]. The consequence is the oxidation of nucleotides, facilitated by an increase in the number of free radicals, which subsequently leads to indirect oxidative damage inflicted upon the DNA [28].

The understanding of oxidative damage in anaerobes is generally limited, with only a few studies primarily focusing on the strict anaerobic model system, *Prevotella melanogaster* [29–31]. These studies indicate that exposure to oxygen results in increased levels of 8-hydroxy-deoxyguanosine (8OHdG), which is a typical marker of oxidative DNA damage [29]. Moreover, it has been demonstrated that these elevated levels of 8OHdG are associated with increased DNA mutations and reduced survival rates [29,31]. To counteract the toxicity of oxygen, microorganisms employ both enzymatic and nonenzymatic protective mechanisms, such as catalases and superoxide dismutase SOD [32,33]. However, certain strict anaerobes, including *P. melanogaster*, exhibit undetectable catalase and SOD activities [29,30].

It is noteworthy that work in our laboratory to isolate DNA from newly identified anaerobic bacteria found in the gut of herbivorous fish for whole genome sequencing [34,35] was very successful when the DNA was extracted anaerobically using the improved methods described here. With this approach, rather than obtaining numerous contigs, we consistently achieved a range of only 1 or 2 contigs, resulting in a genome coverage between 412x to 238x. This contrasts with the recent study by Ref. [4] where, despite implementing a method for high-molecular-weight DNA extraction, the authors achieved a genome coverage of 140x. Our streamlined process significantly simplifies the assembly algorithm. Additionally, we question whether some metagenome studies measuring bacterial compositions present in anaerobic environments, might be impacted by aerobic DNA breakage during extraction. Such a potential distortion in results could lead to a microbial composition analysis which does not wholly reflect the original. This suggests a requirement to assess whether or not anaerobic extraction gives significantly different results.

In conclusion, our study effectively demonstrates the advantages of sampling, storage and conducting an improved extraction protocol in anaerobic conditions. This is evident through the increased DNA yields and improved quality of extracted DNA. Such findings may be valuable for metagenomic studies on other anaerobic communities where the quality and quantity of DNA are crucial for downstream analysis.

CRediT authorship contribution statement

Svetlana Boycheva: Writing – original draft, Methodology, Conceptualization. **Anthony M. Roberton:** Writing – review & editing, Methodology, Conceptualization. **Alessandro Pisaniello:** Writing – review & editing, Methodology. **Bikiran Pardesi:** Writing – review & editing, Methodology. **William Lindsey White:** Methodology, Funding acquisition. **Kendall D. Clements:** Writing – review & editing, Methodology, Conceptualization, Funding acquisition.

Conflict of interest

The authors declare that they have no competing interests.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.anaerobe.2024.102915>.

Data availability

Data will be made available on request.

References

- [1] R.J. Roberts, M.O. Carneiro, M.C. Schatz, The advantages of SMRT sequencing, *Genome Biol.* 14 (6) (2013) 1–4, <https://doi.org/10.1186/gb-2013-14-6-405>.
- [2] M. Jain, H.E. Olsen, B. Paten, M. Akeson, The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community, *Genome Biol.* 17 (1) (2016), <https://doi.org/10.1186/S13059-016-1103-0>.
- [3] B.E. Slatko, A.F. Gardner, F.M. Ausubel, Overview of next-generation sequencing technologies, *Curr. Protoc. Mol. Biol.* 122 (1) (2018), <https://doi.org/10.1002/cpmb.59>.
- [4] C. Penouilh-Suzette, S. Fourré, G. Besnard, L. Godiard, Y. Pecir, A simple method for high molecular-weight genomic DNA extraction suitable for long-read sequencing from spores of an obligate biotroph oomycete, *J. Microbiol. Methods* 178 (2020) 106054, <https://doi.org/10.1016/j.mimet.2020.106054>.
- [5] D.W. Burden, Guide to the disruption of biological samples-2012, *Random Primers* 12 (2012) 1–25. <https://www.researchgate.net/publication/284296370>.
- [6] S.C. Tan, B.C. Yiap, DNA, RNA, and protein extraction: the past and the present, *J. Biomed. Biotechnol.* 2009 (2009) 574398, <https://doi.org/10.1155/2009/574398>.
- [7] Sr. Laskowski, Nucleases: historical perspectives, *Cold Spring Harbor Monograph Arch.* 14 (0) (1982) 1–21. <https://cshmonographs.org/index.php/monographs/article/view/4167>.
- [8] J.H. Martin, D.C. Savage, Degradation of DNA in cells and extracts of the obligately anaerobic bacterium *Roseburia cecicola* upon exposure to air, *Appl. Environ. Microbiol.* 54 (6) (1988) 1619–1621, <https://doi.org/10.1128/aem.54.6.1619-1621.1988>.
- [9] H.J. Flint, A.M. Thomson, Deoxyribonuclease activity in rumen bacteria, *Lett. Appl. Microbiol.* 11 (1) (1990) 18–21, <https://doi.org/10.1111/J.1472-765X.1990.TB00126.X>.
- [10] R.K. Porschen, S. Sonntag, Extracellular deoxyribonuclease production by anaerobic bacteria, *Appl. Microbiol.* 27 (6) (1974) 1031–1033, <https://doi.org/10.1128/AM.27.6.1031-1033.1974>.
- [11] K.S. Johnson, K.D. Clements, Histology and ultrastructure of the gastrointestinal tract in four temperate marine herbivorous fishes, *J. Morphol.* 283 (1) (2022) 16–34, <https://doi.org/10.1002/JMOR.21424>.
- [12] S.H. Duncan, G.L. Hold, H.J.M. Harmsen, C.S. Stewart, H.J. Flint, Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a

- proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov., Int. J. Syst. Evol. Microbiol. 52 (6) (2002) 2141–2146, <https://doi.org/10.1099/00207713-52-6-2141>.
- [13] R.A. Stanley, S.P. Ram, R.K. Wilkinson, A.M. Robertson, Degradation of pig gastric and colonic mucins by bacteria isolated from the pig colon, Appl. Environ. Microbiol. 51 (5) (1986) 1104–1109, <https://doi.org/10.1128/aem.51.5.1104-1109.1986>.
- [14] A.M. Robertson, C.G. McKenzie, N. Sharfe, L.B. Stubbs, A glycosulphatase that removes sulphate from mucus glycoprotein, Biochem. J. 293 (3) (1993) 683–689, <https://doi.org/10.1042/bj2930683>.
- [15] K. Wornell, B. Paredesi, K. Lee, S. Boycheva, A.M. Robertson, W.L. White, High-throughput method for novel medium development for culture of anaerobic gut bacteria, Curr Protoc 2 (7) (2022), <https://doi.org/10.1002/cpz1.463>.
- [16] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, United States, 2001, 4.35, https://books.google.com/books/about/Molecular_Cloning.html?id=Bosc5JVxNpkC.
- [17] K. Wilson, Preparation of genomic DNA from bacteria, Curr. Protoc. Mol. Biol. 56 (1) (2001) 2, <https://doi.org/10.1002/0471142727.MB0204S56.4.1-2.4.5>.
- [18] B. Paredesi, A.M. Robertson, K.C. Lee, E.R. Angert, D.I. Rosendale, S. Boycheva, W. L. White, K.D. Clements, Distinct microbiota composition and fermentation products indicate functional compartmentalization in the hindgut of a marine herbivorous fish, Mol. Ecol. 31 (8) (2022) 2494–2509, <https://doi.org/10.1111/mec.16394>.
- [19] A. Pisaniello, K. Handley, W.L. White, E.R. Angert, J. Boey, K.D. Clements, Host individual and gut location are more important in gut microbiota community composition than temporal variation in the marine herbivorous fish *Kyphosus sydneyanus*, BMC Microbiol. 23 (1) (2023), <https://doi.org/10.1186/s12866-023-03025-2>.
- [20] D.S. Adams, *Lab Math: A Handbook of Measurements, Calculations, and Other Quantitative Skills for Use at the Bench*, second ed., Cold Spring Harbor Laboratory Press, 2014. https://cshlpress.com/default.tpl?cart=1708740097285520379&fromlink=T&linkaction=full&linksortby=oop_title&eqSKUdata=973.
- [21] E. Wnuk, A. Waśko, A. Walkiewicz, P. Bartmiński, R. Bejger, L. Mielnik, A. Bieganski, The effects of humic substances on DNA isolation from soils, PeerJ 8 (2020), <https://doi.org/10.7717/peerj.9378>.
- [22] J. Qin, R. Li, J. Raes, M. Arumugam, K.S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D.R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J.M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H.B. Nielsen, E. Pelletier, P. Renault, S. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, S. Li, M. Jian, Y. Zhou, Y. Li, X. Zhang, S. Li, N. Qin, H. Yang, J. Wang, S. Brunak, J. Doré, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, Consortium MetaHIT, P. Bork, S.D. Ehrlich, J. Wang, A human gut microbial gene catalogue established by metagenomic sequencing, Nature 464 (7285) (2010) 59–65, <https://doi.org/10.1038/nature08821>.
- [23] B.J. Parker, P.A. Wearsch, A.C.M. Veloo, A. Rodriguez-Palacios, The Genus *Alistipes*: gut bacteria with emerging implications to inflammation, cancer, and mental health, Front. Immunol. 11 (906) (2020), <https://doi.org/10.3389/fimmu.2020.00906>.
- [24] T. Yatsunenkov, F.E. Rey, M.J. Manary, I. Trehan, M.G. Dominguez-Bello, M. Contreras, M. Magris, G. Hidalgo, R.N. Baldassano, A.P. Anokhin, A.C. Heath, B. Warner, J. Reeder, J. Kuczynski, J.G. Caporaso, C.A. Lozupone, C. Lauber, J. C. Clemente, D. Knights, R. Knight, J.I. Gordon, Human gut microbiome viewed across age and geography, Nature 486 (7402) (2012) 222–227, <https://doi.org/10.1038/nature11053>.
- [25] J. Kazantseva, E. Malv, A. Kaleda, A. Kallastu, A. Meikas, Optimisation of sample storage and DNA extraction for human gut microbiota studies, BMC Microbiol. 21 (1) (2021), <https://doi.org/10.1186/s12866-021-02233-y>.
- [26] A. Natarajan, A. Han, S. Zlitni, E.F. Brooks, S.E. Vance, M. Wolfe, U. Singh, P. Jagannathan, B.A. Pinsky, A. Boehm, A.S. Bhatt, Standardized preservation, extraction and quantification techniques for detection of fecal SARS-CoV-2 RNA, Nat. Commun. 12 (1) (2021) 5753, <https://doi.org/10.1038/s41467-021-25576-6>.
- [27] N. Chatterjee, G.C. Walker, Mechanisms of DNA damage, repair, and mutagenesis, Environ. Mol. Mutagen. 58 (5) (2017) 235–263, <https://doi.org/10.1002/em.22087>.
- [28] M.J. Xavier, B. Nixon, S.D. Roman, R.J. Aitken, Improved methods of DNA extraction from human spermatozoa that mitigate experimentally-induced oxidative DNA damage, PLoS One 13 (3) (2018), <https://doi.org/10.1371/JOURNAL.PONE.0195003>.
- [29] T. Takeuchi, Y. Nakaya, N. Kato, K. Watanabe, K. Morimoto, Induction of oxidative DNA damage in anaerobes, FEBS Lett. 450 (3) (1999) 178–180, [https://doi.org/10.1016/S0014-5793\(99\)00497-4](https://doi.org/10.1016/S0014-5793(99)00497-4).
- [30] T. Takeuchi, N. Kato, K. Watanabe, K. Morimoto, Mechanism of oxidative DNA damage induction in a strict anaerobe, *Prevotella melaninogenica*, FEMS Microbiol. Lett. 192 (1) (2006) 133–138, <https://doi.org/10.1111/j.1574-6968.2006.tb09371.x>.
- [31] S. Takumi, M. Komatsu, K. Aoyama, K. Watanabe, T. Takeuchi, Oxygen induces mutation in a strict anaerobe, *Prevotella melaninogenica*, Free Radic. Biol. Med. 44 (10) (2008) 1857–1862, <https://doi.org/10.1016/j.freeradbiomed.2008.02.003>.
- [32] B. Halliwell, J.M.C. Gutteridge, The antioxidants of human extracellular fluids, Arch. Biochem. Biophys. 280 (1) (1990) 1–8, [https://doi.org/10.1016/0003-9861\(90\)90510-6](https://doi.org/10.1016/0003-9861(90)90510-6).
- [33] H. Sies, Oxidative stress: oxidants and antioxidants, Exp. Physiol. 82 (2) (1997) 291–295, <https://doi.org/10.1113/EXPPHYSIOL.1997.SP004024>.
- [34] B. Paredesi, A.M. Robertson, E.M. Wollmuth, E.R. Angert, D.I. Rosendale, W. L. White, K.D. Clements, *Chakrabartyella piscis* gen. nov., sp. nov., a member of the family *Lachnospiraceae*, isolated from the hindgut of the marine herbivorous fish *Kyphosus sydneyanus*, Int. J. Syst. Evol. Microbiol. 73 (10) (2023), <https://doi.org/10.1099/IJSEM.0.006100>.
- [35] B. Paredesi, A.M. Robertson, E.M. Wollmuth, E.R. Angert, D.I. Rosendale, W. L. White, K.D. Clements, *Tannockella kyphosi* gen. nov., sp. nov., a member of the family *Erysipelotrichaceae*, isolated from the hindgut of the marine herbivorous fish *Kyphosus sydneyanus*, Int. J. Syst. Evol. Microbiol. 72 (5) (2022), <https://doi.org/10.1099/IJSEM.0.005374>.