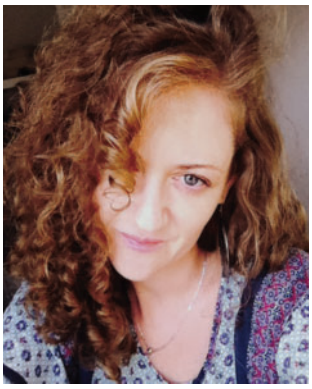
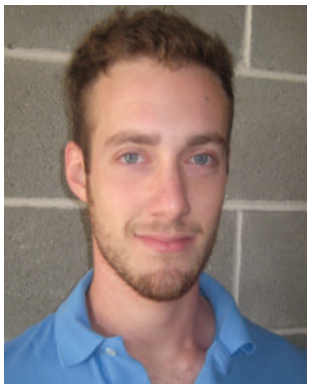
Wastewater, wheat and table wipes: adventures in culture-independent microbiology


Jacob E Munro[A], *Deborah J Rich*[A], *Simon Dingsdag*[A] *and Nicholas V Coleman*[A,B]

[A]School of Molecular Bioscience, Building G08, University of Sydney, Darlington, NSW 2006, Australia.

[B]Corresponding author. Tel: +61 2 9351 6047, Fax: +61 2 9351 5858, Email: nicholas.coleman@sydney.edu.au

The sequencing of ribosomal RNA and DNA (rRNA/rDNA) from environmental samples heralded a new age in microbiology[1–3]. The advent of next-generation sequencing supercharged these methods, which now give high-resolution data sets, enabling real insights into microbial diversity and function in complex systems[4–7]. Here, three local applications of 16S rDNA pyrosequencing are described, which highlight the usefulness of this approach for addressing practical questions in diverse areas of microbiology. Limitations of the sequence-based approach will also be discussed.

Wastewater: understanding the shutdown response in biological aerated filters

An industry partner approached our lab for assistance with managing microbes in the biological aerated filter units (BAFs) in their wastewater treatment plant. The BAFs were designed to degrade volatile fatty acids (VFAs) in the wastewater stream, but were problematic after 'shutdown' events. Such events involve stopping the water flow for many days, and in some cases cleaning the BAFs; these actions change the physiology and/or community in the BAFs such that they do not readily re-start VFA oxidation.

We sampled the BAF material (zeolite+biofilm) at intervals over a time course spanning both shutdown and restart events. RNA was extracted, and reverse-transcribed to cDNA, then used for 16S and 18S rRNA gene PCRs, and tag-pyrosequencing. We used bead-beating combined with a commercial RNA extraction kit to good effect; this was an 'easy' template due to the abundance of biomass in the BAF material (Figure 1). Our rationale for using ribosomal RNA as the template was that this is a better marker for the *activity* of microbes compared to ribosomal DNA, which is better correlated to cell *abundance* (e.g. see Hunt *et al.*[8]).

Distinctive changes in the BAF community occurred during shutdown (representative data from one of three replicate BAFs are shown in Figure 2); *Arcobacter*, *Zoogloea*, and *Bdellovibrio* declined, while *Rubrivivax* and *Pedomicrobium* increased. After restart of flow, the community seemed to return to the initial structure, but this robust response to perturbation at the genus level did not correspond to success in restarting VFA oxidation (data not shown). The changes involved in the shutdown response may be occurring at finer-scale taxonomic resolution, or might not involve ribosome abundance (e.g. they may involve enzyme induction).

Bdellovibrio is a predator on other bacteria. This genus suffered dramatic declines in rRNA abundance after shutdown (50- to 150-fold). It is interesting that this 'top predator' was the most sensitive to ecosystem disturbance – this mirrors patterns seen in macro-organisms[9]. *Bdellovibrio* may be a predator of a bacterium that is inhibitory to VFA oxidation, or it may be acting here as an

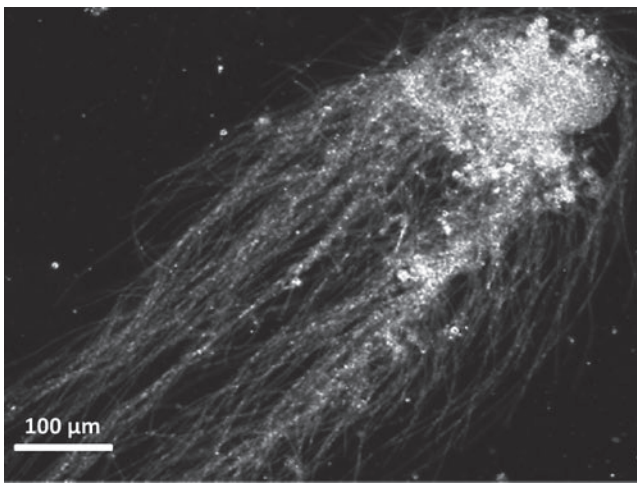


Figure 1. Microscopy of BAF biomass floc (acridine orange stain). The abundant filamentous cells that define these flocs could be *Sphaerotilus* and/or *Thiothrix*.

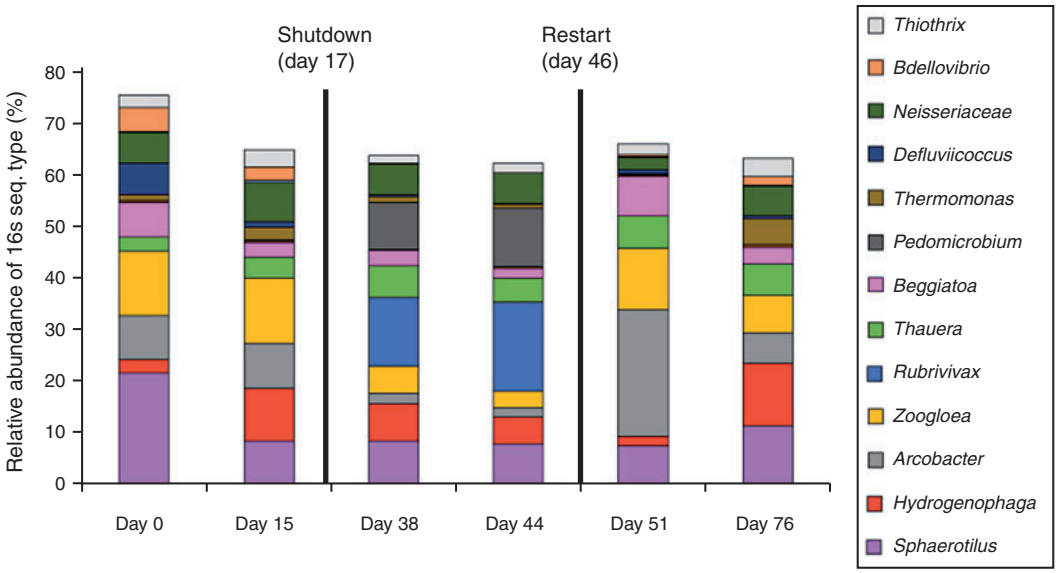


Figure 2. Bacterial community response to BAF shutdown and restart, as inferred from 16S rRNA relative sequence abundances.

indicator organism of the chemical changes in the system. Further work is needed to elucidate the microbial basis of the BAF shutdown response.

Wheat: tracking inoculant strains and discovering indigenous microbiota

Ethylene (C_2H_4) is a gaseous plant hormone[10]. Ethylene-oxidising bacteria can be readily isolated from soil[11] – do these bacteria interact with plants based on the ethylene system? Alternatively, these bacteria could be consuming ethylene made by microbial fermentation[12]. . .the jury is out. Ethylene-oxidising isolates are nearly always fast-growing *Mycobacterium* spp.[13,14] – these are a fascinating group of microbes, which are mostly non-pathogenic, but highly immunogenic[15]. They may even play a role in influencing our moods[16,17].

We have begun a study to investigate the interactions of ethylene-oxidising bacteria and plants. The 16S pyrosequencing approach was used to provide information on the persistence of the inoculant strains and to reveal which types of indigenous bacteria were present. Note that although the tag-pyrosequencing data are not quantitative in the sense that sequences do not correlate 1 : 1 with cells, the data can be used to discern trends in relative abundances, and provide a reference point for viable counts in the case of the inoculant strain.

Preliminary data from two individual wheat plants (control/inoculated) are shown in Table 1. Note that the DNA extraction method used here has captured both the surface microbiota and the endophytes in the wheat plants, as evidenced by the abundant chloroplast sequences that are recovered (green highlight). These organelles contain their own 16S rDNA, which bears testament to their cyanobacterial ancestry[18]. At this stage it is not clear which of these taxa are surface microbiota, and which are endophytes.

The tag-pyrosequencing approach easily detected our inoculated ethylene-oxidising bacterium (yellow highlight), but intriguingly, the sequence data also revealed an indigenous *Mycobacterium* species (blue highlight) – this was the 6th-most abundant bacterial sequence detected in the uninoculated plants. Closer inspection of this sequence reveals that the indigenous *Mycobacterium* was closely related to species known as ethylene-oxidisers. Is this organism involved in ethylene oxidation *in vivo* in the wheat plant?

Table 1. Relative abundance (%) of bacterial 16S rRNA gene sequences in inoculated and control wheat plants.

Control		Inoculated	
Mezorhizobium	63	*Ralstonia*	41
Ralstonia	25	*Mezorhizobium*	39
Acinetobacter	2.9	*Mycobacterium*	7.8
Xanthobacter	2.8	*Xanthobacter*	3.5
Chloroplast	1.5	*Acinetobacter*	2.3
Mycobacterium	0.4	*Acidovorax*	0.8
Variovorax	0.3	Chloroplast	0.8
Acidovorax	0.3	*Pseudomonas*	0.6
Pseudomonas	0.2	*Variovorax*	0.6
Ralstonia	0.2	*Mycobacterium*	0.5

Wipes: assessing risks from bacteria in a shopping centre food court

Our lab was contacted by a union representing cleaners to undertake an investigation into the microbiology of the shopping centre food court. There was concern from the union that the cleaners were under-resourced, based on rumours of poor practices such

as re-using cleaning cloths for excessive lengths of time, or using the same cloths in multiple places (e.g. bathrooms and food court).

We obtained samples of a cleaning cloth used in the food court, and also table-wipes from many individual table surfaces. Our aims were to determine the total bacterial numbers (plate count), to determine if pathogenic bacterial types were present, and to determine if faecal indicator organisms (*E. coli*) were present. These data would allow us to estimate firstly the level of public health risk, and secondly, to see if there was evidence for cross-area usage of cloths between food court and bathroom (for full study details, see Dingsdag[19]).

As part of this study, we wanted to get a sense of the relationship between the bacterial types growing on the agar used (R2A), and the total community in the cleaning cloth – are the isolates grown on agar really representative of the major types present in this environment? This once intractable-question is now easy to answer by doing a 16S PCR on DNA extracted direct from the cloth, and another PCR on DNA extracted from the pooled colonies grown on agar, and pyrosequencing both PCR products (Figure 3).

The majority of bacterial types detected on agar plates were consistent with those detected by direct DNA extraction from the cleaning cloth, although their relative order changed. *Aeromonas* was the exception; this was third-most abundant genus in the cloth, but did not grow at all on R2A. This could be due to inhibition by other faster-growing bacteria, since aeromonads can certainly be cultivated on R2A[20].

The ease of culturability of most bacteria in the cloth could be because this is a eutrophic environment (like culture media), or perhaps this habitat selects stress-resistant types (detergents, heat); these may also resist the stress of isolation on agar.

The most abundant sequences detected in the cleaning cloth were from genera that include human pathogens, such as *Stenotrophomonas*, *Acinetobacter*, and *Aeromonas*. While tag-pyrosequencing (~400 bp sequence) cannot reliably identify bacteria to the species level, the closest sequence matches in many cases (>99%) were to pathogens such as *S. maltophilia*, *Ac. baumannii* and *Ae. hydrophila*. Both coliforms (*Enterobacter, Citrobacter*) and faecal coliforms (*E.coli*) were detected, with *E.coli* at 0.3–0.5% of sequences – this may indicate the use of this cloth in the bathroom, but could also be due to poor hygiene of the general public, who contribute to the bacterial load here.

Limitations of the tag-pyrosequencing approach

Any type of PCR will be limited by the primers used. The 'universal' primers for targeting 16S and 18S rDNA are not identical to all-prokaryote or all-eukaryote ribosomal sequences, respectively, and they cannot amplify all of the sequence types in a complex habitat[21]. Further, different sequence types will be amplified with differing efficacy, if present in a mixed DNA template. The latter effect is marked, and can be demonstrated by PCR and sequencing of defined mixtures of a few dozen ribosomal sequences[22]. Another serious problem with PCRs from mixed templates is the generation of chimeric sequences, which need to be specifically detected and removed[23].

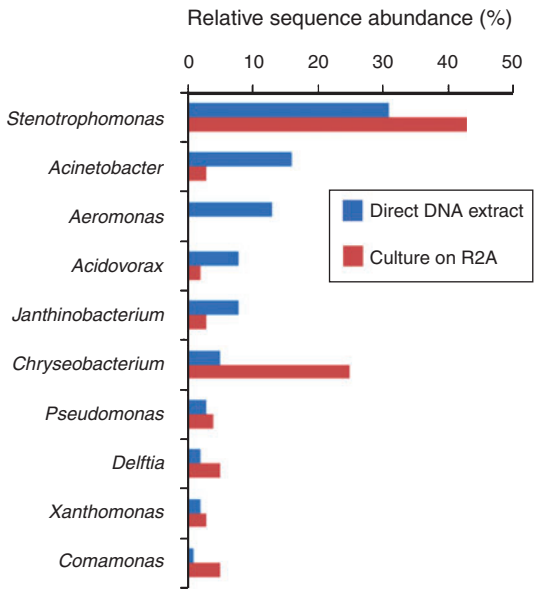


Figure 3. Comparison of culturable vs total bacterial communities in a cleaning cloth, based on 16S rDNA sequence abundance. Sequences are ranked based on abundance in the direct DNA extract (blue bars).

Biases also arise from nucleic acid extraction, since different microbes are lysed with different efficacy. Physical disruption (e.g. bead-beating) is often the method of choice, since it is rapid, and it can lyse both bacteria and eukaryotes, but neither this method or the alternatives are guaranteed to lyse all microbial types, which leads to a bias towards the more easily lysed types in sequence libraries[24,25]. The peculiarities of different environmental matrices (e.g. soils vs. clinical samples) impact strongly on the yield and purity of extracted DNA, and its usefulness for downstream amplification steps[26]. This is a particular challenge for forensic use of tag-pyrosequencing, where legal decisions are made based on sequence data[27].

Pyrosequencing is more error-prone than Sanger sequencing, and the level of errors generated can be sufficient to yield false operational taxonomic units (OTUs) if rigorous sequence quality control is not employed. This means that unique clones in sequence data may be genuine members of the rare biosphere, or they may be sequencing errors[28,29]. Another pyrosequencing artefact is the generation of false clusters of identical or closely related sequences – these are present at up to 35% in some metagenomic datasets[30].

Many traps in tag-pyrosequencing analysis relate to over-interpretation of the data[31] – this could include extrapolating cell numbers from numbers of rDNA sequences (rRNA gene copy number varies in different phyla), postulating physiological functions based on ribosome sequences (most bacteria have highly variable metabolism and physiology), or mistaking statistical correlation s between sequences as causal linkages (an error in logic).

Concluding remarks

Environmental microbiologists have an important role to play in addressing humanity's major challenges in the 21st Century. Our technical ability to attack these problems is more powerful than ever, but our efforts locally are constrained by a lack of funding and a

lack of vision from our large institutions and governments. As a Society, and as individual microbiologists, we need to push harder for recognition of the reality and seriousness of environmental problems, and the importance of microbiologists in solving these problems.

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Biographies

Nick Coleman gained a PhD (Microbiology) from USyd (2000), and worked as a postdoc at Tyndall Air Force Base (USA, 2000–2003) and at USyd (2003–2006), then began as a Lecturer at USyd in 2006. Nick is a member of the ASM NSW Branch Committee and the ASM2015 Canberra Local Organising Committee.

Jacob Munro completed his BSc(Adv) at USyd in 2011, graduating with Honours in Microbiology. He is currently studying for a MSc (IT-Bioinformatics) degree at UNSW. Jake's research interests include bioinformatics, biotechnology and microbial ecology.

Deborah Rich studied a BSc(Adv) at USyd, graduating in 2014 with Honours in Microbiology. Deb is starting a PhD in the Coleman lab, studying the interactions of plants and ethylene-oxidising-bacteria, and the potential uses of these bacteria for delaying ripening and spoilage.

Simon Dingsdag did his BSc at USyd, graduating in 2010 with Honours in Microbiology. Simon is currently studying for his PhD (USyd) at the Westmead Centre for Dental Research. His research interests include microbial ecology and oral microbiology.