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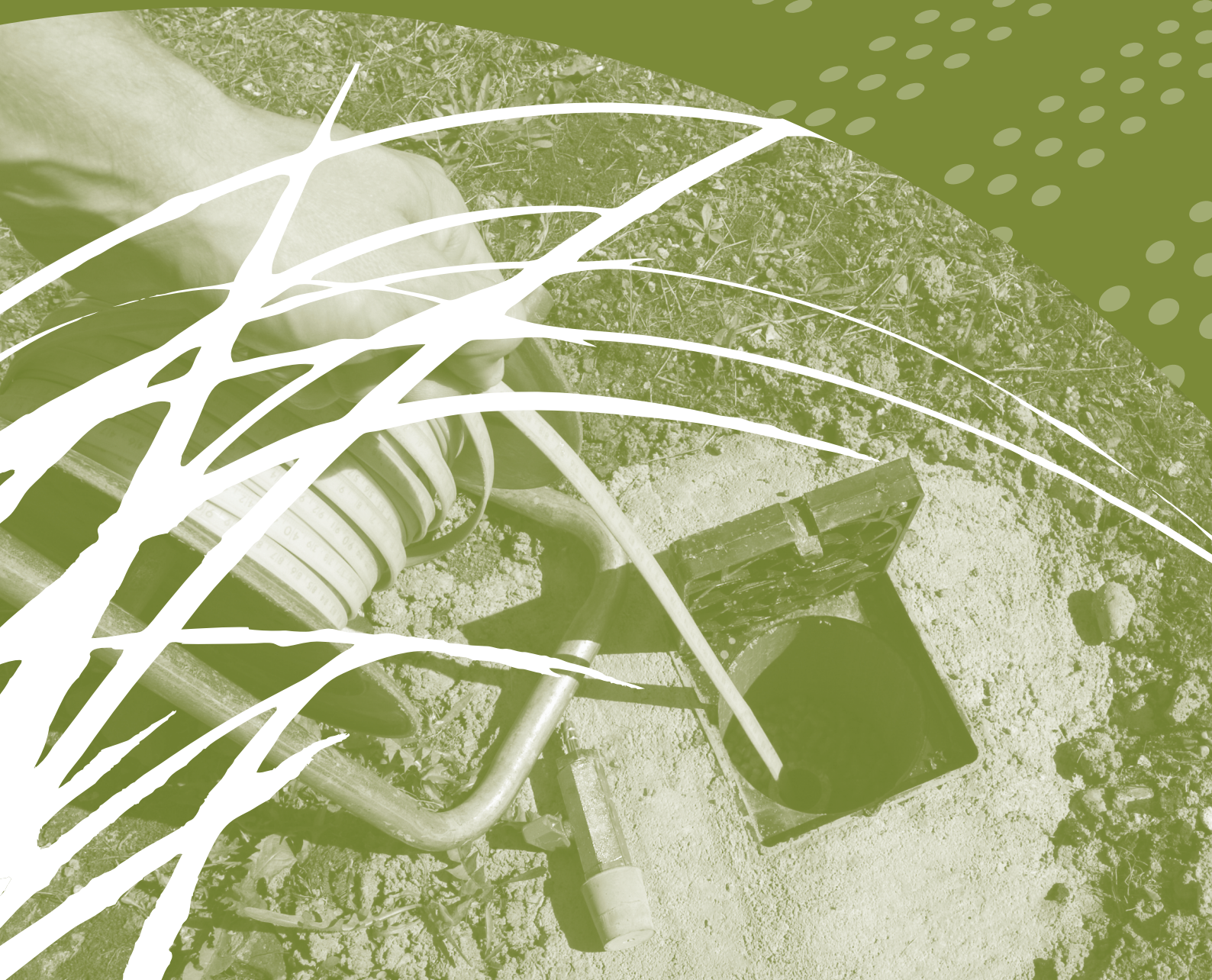
no. 21

technical report

Sampling strategies for biological assessment of groundwater ecosystems

G.C. Hose and M.J. Lategan

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G. C. Hose[1] and M. J. Lategan[2]

[1] Macquarie University, [2] University of Technology Sydney

This report should be cited as:
Hose, GC & Lategan, MJ 2012, *Sampling strategies for biological assessment of groundwater ecosystems,* CRC CARE Technical Report no. 21, CRC for Contamination Assessment and Remediation of the Environment, Adelaide, Australia.

Executive summary

The effective management of groundwater resources across Australia is essential to meet current and future national water needs. At the same time, the significance of groundwater ecosystems in terms of their biodiversity and ecosystem services is increasingly being recognised such that surveys of groundwater ecosystems are now often a part of development applications and environmental impact assessments.

Despite the growing awareness of the value of groundwater ecosystems, there is currently little guidance available to assist practitioners in their assessment. Accordingly, the aim of this document is to provide guidance on methods and strategies for the biological assessment of groundwater ecosystems, specifically in the context of localised environmental threats or impacts. Within the context of contaminated site assessment, routine investigation of groundwater ecosystems may not be required, but should be considered in areas of ecological significance or conservation value.

The groundwater environment is characterised by total darkness. As a result, there are no photosynthetic primary producers and (usually) only low concentrations of organic carbon as an energy source for the ecosystem. The biota of the ecosystem is comprised of two major components: the microbes (including bacteria and fungi), and the larger mostly crustacean macro- and meiofauna (stygofauna). Accordingly, groundwater ecosystems are very different from surface water ecosystems, and so require different strategies for their biological assessment.

Sampling of stygofauna is generally conducted by means of pumps, nets or traps, with the choice of method often having little impact on the variety of animals collected, but some influence on the abundance of those animals. Importantly, to adequately assess the diversity of stygofauna at a location, multiple bores must be sampled on multiple occasions. Samples from multiple bores on a single occasion, or from a single bore on multiple occasions, will not adequately assess stygofauna diversity. Our sampling indicates that at least five sampling locations and five sampling events may be required.

Microbial assemblages may be assessed by a variety of means including molecular or metabolic fingerprinting, direct measurement of biomass and microbial enzyme activity. Irrespective of the method chosen, repeat temporal and spatial sampling should be undertaken. While measures of microbial activity at relatively undisturbed sites may be variable over time, the effects of disturbance to an aquifer may cause a large and readily detectable shift in microbial activity, greatly exceeding the spatial and temporal variability among undisturbed sites.

Assessments of aquifer ecosystems in the context of environmental impact assessment should examine both microbes and stygofauna, reflecting the major biotic components of the ecosystem. Multiple samples over space and time are necessary, with the exact level of replication and sampling effort ideally determined by site-specific studies.

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Preface

The assessment of groundwater ecosystems is increasingly being required by governments and regulators in Australia with respect to development applications. The management of contaminated sites, however, does not often have these same requirements.

Assessment of groundwater ecosystems may not be necessary for routine contaminated site investigations, but should be encouraged in situations where groundwater contamination poses a risk to areas of ecological significance and conservation value. However, assessments of groundwater ecosystems can also be useful even in routine contaminated site assessment by providing an ecological context for remediation targets, or establishing environmental harm resulting from contamination.

1. Aims and scope

The purpose of this document is to provide guidance to practitioners on the assessment of aquifers for the presence and activity of groundwater biota. Specifically, the document considers the sampling of aquifers for the purpose of environmental impact assessments or similar studies, particularly in the context of localised groundwater contamination. The revised NEPM (2011) Schedule B2 provides detailed guidance on the sampling of groundwater for site characterisation, with a focus on contamination assessment. Critical in that process is the determination of data quality objectives (DQO), i.e., an *a priori* assessment of the data needs for a study. This document serves as an addendum to the NEPM (2011) Schedule B2 by providing specific guidance on groundwater sampling for biological assessment, where the need for such information is identified in the DQO process.

This document provides a summary of sampling methods based on studies reported in the scientific literature, and those completed recently as part of a CRC CARE and NSW Environmental Trust funded project. The document provides evidence-based guidance on sampling strategies to investigate the presence and diversity of groundwater fauna, and both stygofauna and microbial assemblages.

The information provided here is illustrative and for guidance only. It does not, and should not, replace the need for site-specific investigation of groundwater ecosystems.

This document is limited in its discussion to subterranean aquatic ecosystems containing stygofauna. It does not address issues of sampling subterranean terrestrial fauna (troglofauna). For information on troglofauna, and additional advice on stygofauna assessment, please see WA EPA (2007).

2. Background – the nature of groundwater ecosystems

Groundwater ecosystems differ greatly from surface environments, in terms of both biota and driving ecological variables and processes. There is no light, and consequently there are usually no primary producers in groundwater ecosystems (except for occasional chemoautotrophic organisms). The ecosystem is thus largely dependent on allochthonous carbon that filters from the surface. Carbon utilised by bacteria and fungi provides the basis of the aquifer food web (Boulton 2000). In many cases, where conditions permit, higher order invertebrates are also present. These include meiofauna such as turbellaria, rotifera, nematoda and protozoa (Humphreys 2006) and macroinvertebrates (predominantly crustaceans) such as copepoda, syncarida, amphipoda, isopoda and ostracoda. Insects are relatively uncommon (Humphreys 2006). Vertebrates are rare (occasionally found in karstic aquifers, with only two species in Australia), constrained by the often small void spaces in the subterranean sediment/groundwater matrix.

The supply of external carbon limits the productivity of groundwater ecosystems, usually constraining pristine groundwaters to be low-energy environments with low biomass and abundance, and patchy distribution of biota. Despite relatively stable physical conditions of temperature and water quality, groundwater ecosystems are characterised by biotic heterogeneity, meaning that there is an uneven distribution of biota over space and time. It is likely that this is driven by the uneven and patchy distribution of microbes that form the basis of the trophic chain. As a result, sampling and collection of groundwater fauna from bores can be very 'hit or miss'.

With the absence of primary producers (such as plants or algae that cannot grow in the dark) and vertebrates, groundwater ecosystems tend to be relatively simple systems. In terms of meiofauna, these are typified by low α diversity (few species at any one locality) and have a 'truncated' functional and taxonomic diversity (Gibert and Deharveng 2002). This creates a system with (generally) low horizontal (within trophic level) and vertical (between trophic level) diversity (*sensu* Duffy et al. 2007) in a given location, and concomitant short food chains. However, isolation has created a fauna dominated by short-range endemic species, providing high ß diversity (many species across localities) of invertebrates (Humphreys 2008). This trend is not evident in microbial assemblages which appear to be more widely distributed (Griebler and Leuders 2009).

With increasing awareness of the significance and diversity of groundwater ecosystems, and their consideration in regulatory processes, the assessment of groundwater ecosystems is increasingly required as part of environmental impact assessments. There is growing evidence of the inter-seasonal variability in groundwater assemblages (Hancock and Boulton 2009), and also differences in biota between aquifers (Hancock and Boulton 2008; Hahn and Fuchs 2009). However, there is little data available on small-scale spatial and temporal variability of groundwater biota, i.e., variation within aquifers from metres to kilometres and from week to week. Importantly, with respect to environmental assessment, it is at these scales that much environmental impact assessment is likely to be conducted.

In summary, the attributes of groundwater ecosystems that are likely to influence how they will be assessed are:

- there is frequently few sampling points (bores)
- species are cryptic
- fauna are heterogeneously distributed
- fauna are typified by species with very localised distributions, and
- microbial and macroinvertebrate assemblages are the key biotic groups.

3. Sampling methods for subterranean aquatic biota

3.1 Sampling bores and locations

Natural access to groundwater ecosystems is limited to springs, caves, sinkholes or seeps where groundwater is accessible at the surface. These natural features provide few windows into the subterranean world and are generally insufficient to provide a replicated and targeted groundwater sampling program. Accordingly, the construction of bore holes or wells is often needed to facilitate sampling. It is beyond the scope of this document to provide advice on the design of bores and fields for groundwater sampling (interested readers should see Sundaram et al. (2009) and NEPM (2011, Schedule B2)). Instead, this document provides guidance on the sampling requirements for a robust assessment of groundwater ecosystems. Such information should be considered where a bore field is to have the dual purposes of water quality monitoring (see Sundaram et al. (2009)) and ecological assessment.

The construction of bores and wells is generally achieved by drilling into the subsurface geological strata until the desired depth is reached. Groundwater may occur at several discrete levels below ground, and bores should target the specific level of interest. Depending on the geology, bore holes may be open, or lined with PVC or metal casings. Often, the casings contain slots at specific depths to allow water to enter the bore hole as a means of targeting groundwater at a specific depth. Casings may also be covered by a fine mesh screen designed to keep fine particles (silt/sediment) from the bore, particularly if a bore is used for water supply. The size of the slots and any mesh screens will dictate whether or not groundwater fauna can enter the bore hole. Mesh sizes less than 1 mm may be prohibitive to the entry of some invertebrate taxa. The mesh size and construction of the bore should be considered before deciding whether any existing bores will be suitable for assessing stygofauna.

The casing material and age of the bore may also influence the collection of stygofauna. Steel or PVC cases may be used in bore construction, with the latter now more commonly used – in part because of the deterioration of steel-cased bores over time. Although there is no empirical data comparing the two materials, anecdotal evidence suggests that greater richness and abundance of stygofauna are likely in bores with PVC casings (Hancock pers. comm.). As steel-cased bores tend to be older, the comparison of bore construction may be confounded by bore age.

The disturbance of an aquifer associated with bore construction is likely to also disrupt the aquifer biota. Accordingly, it is recommended that bores sampled are at least 6 months old (WA EPA 2007). If this is impractical, bores greater than 3 months old may be sampled repeatedly (WA EPA 2007).

Local hydrogeology can complicate stygofauna sampling, particularly where multiple aquifers are present. Across a study area it is important to ensure that the same aquifer is being sampled from different bores. While sampling from similar depths may achieve some certainty here, comparison of water quality variables may also inform sampling. Importantly, sampling should not be done without some understanding of the local hydrogeology, otherwise sampling efforts may be wasted.

3.2 Groundwater sampling and purging

Frequently, best practice for groundwater sampling for water quality assessment requires that bores must be purged (i.e. have all standing water removed and replenished) before a sample is collected for chemical analysis. However, purging a bore before sampling is not desirable for stygofauna sampling. Instead, samples of stygofauna may be collected straight from the bore without purging. In this way, the bore can be viewed as a trap for stygofauna, with samples reflecting a time-integrated 'catch' of fauna. However, for microbial sampling, the bore should be purged prior to sample collection. This is conducted for the same reason as chemical sampling – to ensure that the sample reflects that in the surrounding aquifer. If using a high flow pump, this requires removal of 3 – 5 bore volumes (Sundaram 2009). If using a low flow pump, this may be achieved once physicochemical properties of the groundwater have stabilised (NEPM 2011). The routine measurement of pH, electrical conductivity (EC), temperature, dissolved oxygen (DO), oxidation-reduction (redox) potential (Eh) and alkalinity is recommended in tandem with all stygofauna and microbial sampling.

3.3 Sampling methods for stygofauna

There are a variety of methods for sampling stygofauna, grouped loosely as pump methods, net methods or trapping methods. Most studies show few differences in the richness of stygofauna in samples collected using the various methods (Dumas and Fontanini 2001; Hahn 2005; Allford et al. 2008; Hancock and Boulton 2009).

It is important when conducting stygofauna sampling that the bore is not purged before the sample is collected, unless it is a specific aim of the study to do so. Often, stygofauna and water quality sampling will be undertaken in the same bores as part of the same study. It is important that operators collecting those samples communicate and, ideally, collaborate. The majority of invertebrates collected in a stygofauna sample are those in the bore hole at the time of sampling (rather than being in the surrounding aquifer). Purging a bore removes animals, which may take weeks or months to re-establish. Frequent or repeated purging for water quality sampling can thus interfere with stygofauna sampling, and every effort should be made to coincide these activities.

Studies limited to stygofauna assessments have frequently employed net sampling (using modified plankton nets) to collect animals from bores. These nets with narrow openings and fine (>50 μm) mesh are repeatedly hauled through the water column (Figure 1). The advantages of using this approach are that it offers a relatively cheap and simple means of sampling, and appears a reliable means of sampling stygofauna relative to other methods available. Following the WA EPA (2007) guidelines, a minimum of 6 net hauls should be collected, with three hauls of a fine mesh net (50 – 63 μm mesh) and three hauls of a coarser mesh net (100 – 150 μm mesh). There is unlikely to be much difference in the sampling efficiency of the 50 and 63 μm mesh nets; however, 50 μm mesh should be used where possible in order to standardise sampling approaches.

Figure 1. Weighted plankton nests of various diameters and mesh sizes used for sampling stygofauna.

Bailers are frequently used to collect groundwater samples, but can also be used for stygofauna collection. Bailers are best suited to shallow, small volume bores where nets and pumps can be difficult. Bailers are particularly useful for sampling perched aquifers and swamps. An advantage of using bailers is that they can disturb and collect fauna directly from the sediment at the bottom, a task which is difficult with pumps and nets. Bailers are also effective at sampling the entire bore contents. When using bailers, operators should decide on a desired sample volume and this should guide the sampling effort, rather than the number of hauls.

Traps are a low-cost means of collecting stygofauna. Traps – baited or unbaited – are deployed in bore holes where they are left for varying periods of time, and are later retrieved. The fauna collected appears taxonomically similar to that collected by other means, but traps provided greater richness and abundances of fauna compared to pumped samples (Hahn 2003 in Hahn 2005; Scarsbrook and Halliday 2002). Traps also allow for depth stratified sampling (Hahn 2005), a task not easily achieved with other approaches. However, traps do require repeat visits for deployment and collection.

Apart from netting, pump methods appear to be the most widely used method. Water is removed from the bore hole by way of a mechanical pump, and then filtered to remove animals from the liquid (Figure 1). As mentioned above, bores are generally not purged before sample collection. Volumes pumped vary from 2 L to 1000 L (Malard et al. 1997) but there appears, at least in Australia, to be a tendency towards volumes in the order of 300 L (Hancock and Boulton 2009; Hose unpub). WA EPA (2007) suggests that 3 bore volumes or 300 L (whichever is greater) is removed for sampling. To achieve consistency, pump sampling should aim to remove 300 L of groundwater or 3 bore volumes, whichever is greater. Samples of this volume may be difficult to achieve in slowly replenishing bores. Importantly, the volume of sample collected and the bore volume should always be documented.

Pump methods can be time consuming, and are dependent on the capacity of the pump and the replenishment rate of the bore – the latter of which may be initially unpredictable. In cases where discharge is low and pumping rate slow, smaller

volumes of water may be collected. This may still provide a suitable representation of the local biodiversity, as most fauna are collected early in the sampling period, with the richness and abundance of stygofauna collected diminishing as more water is pumped (Hancock and Boulton 2009). However, the decline in richness and abundance of stygofauna during sampling may not be linear, which can complicate the extrapolation of abundance and richness estimates in full samples from partial samples. It is difficult or impossible to collect discrete depth samples with pump methods, even when sampling level is constrained by the use of packers, because water can be drawn into the bore from a capture zone in the surrounding aquifer that may exceed the target sampling depth.

The pump type to be used will also be an important consideration for sampling. Critically, the pump should not damage the animals collected, which is a potential disadvantage of impeller driven pumps, and a clear advantage of pneumatic and inertia type pumps. Pump rate also influences the fauna collected, and should be standardised as much as possible between samples, but this may be difficult in bores with low rates of replenishment. Higher pump rates presumably have a stronger 'suction' and ability to draw animals in from the surrounding aquifer, leading to potentially a greater number of animals being collected. WA EPA (2007) recommends a pump rate of 10 L/min. This can be readily estimated by recording the time taken to fill several buckets.

Low-flow pumps that are often used for chemical sampling of groundwater appear rarely used for stygofauna sampling, and so their efficacy in this role has not been tested. As a result, a 'high-flow'-style pump is recommended for stygofauna use sampling. Bearing in mind the potential importance of pump rate in influencing the collection of stygofauna, estimates of pump rate should be made at the time of sampling.

Figure 2. An inertia pump set up for groundwater extraction - filtering groundwater samples after pumping to collect stygofauna.

Photos taken by K Korbel.

Water pumped from an aquifer, or collected in nets and bailers, may contain invertebrates – albeit often in a low density. These samples should be filtered to concentrate the invertebrates, and facilitate storage and sample processing. This may be best achieved by passing the water collected from the bore through a fine mesh sieve and retaining the contents of the sieve for later processing. The mesh size of the sieve used will set the maximum size of the invertebrates collected as smaller animals will pass through the sieve. Mesh size of 63 μm is commonly used (cf. Hancock & Boulton 2009); however, 50 μm mesh sieves are also recommended (WA EPA 2007). Although there is little difference in the sampling efficiency between the 50 and 63 μm mesh size (Hose unpub data), 50 μm mesh should be used where possible in order to standardise sampling approaches between studies.

Depending on the purpose of the sampling, invertebrate samples may be preserved or retained live for later analysis. Preserving samples in 100% ethanol effectively preserves the specimens and DNA, allowing later molecular (genetic) analysis. Dilute (70%) ethanol may also fulfil this purpose, but with the likelihood that the sample already contains some water, already diluted ethanol may be further diluted beyond its effectiveness. It is important when preserving samples to ensure that any preservative is distributed throughout the sample. This may require agitating the sample to ensure adequate dispersal, particularly in samples containing large amounts of sediment.

A flow chart for stygofauna sampling methods is provided in Appendix 1. In the discussion of sampling strategies below an inertia type pump was used to remove (generally) 300 L of groundwater which was filtered using a 63 μm mesh sieve (cf. Hancock and Boulton 2009). Samples were preserved in 100% ethanol to facilitate later molecular analysis, and rose bengal stain was added to assist in sample processing.

3.4 Processing samples for stygofauna assessment

Typically, field-sampling of stygofauna will result in a sample containing stygofauna and varying amount of sediment. The first step in the analysis of this sample is the separation of the invertebrates from the sediment. The small size and lack of pigmentation of most stygofauna means they can be difficult to see, often making this process difficult. The addition of a stain to the sample prior to processing can aid in the identification of the invertebrates in the sediment. Rose bengal stain has been used effectively for this purpose. This stain makes biological material pink in colour, providing good contrast between invertebrates, and pale sand and silt or dark organic matter in the samples. For best results, the stain should be added to the sample a couple of days prior to sample processing.

Invertebrate samples should be processed using a dissecting microscope with approximately 60x magnification. When processing, samples should be decanted into a sieve with the same mesh size as was used in sample collection. The preservative should be washed from the sample, and the sieve contents transferred to a petri dish (or similar). For ease of processing, the sediment in the petri dish should be inundated; tap water is sufficient here. The sediment should then be systematically sorted, and any invertebrates removed. Invertebrates removed should be placed into a labeled vial containing preservative, ideally 100% ethanol. For this sorting process, greatest efficiency and accuracy is achieved by processing the sediment in a number of small

portions rather than one or a few larger portions. Whether it is necessary to remove all individuals from a sample will be determined by the DQO; in particular, whether the sample is to be of a qualitative or quantitative nature.

The removal of stygofauna from other sampling debris can be a labour-intensive task. In some cases, this sampling effort can be reduced by the use of flotation techniques in which samples are mixed with dense sugar or salt solutions, causing fine organic material (such as small stygofauna) to float and separate from heavier sample debris (e.g. Barmuta 1984; Hall et al. 1996). This allows the stygofauna to be easily removed. This process is useful for separating large numbers of small specimens from samples, but the sample still needs to be checked as it will not separate all animals from the debris. Furthermore, it has not been tested whether the solutions used in the flotation interfere with the later DNA extraction.

Stygofauna may be reliably identified to a coarse taxonomic level (class, order level) by operators with expertise in the identification of aquatic invertebrates. However, identification to family level or below is, in most cases, the domain of taxonomic experts. The cost of, and time needed for, expert identifications should be factored into projects where such information is needed. This should be considered in the DQO process (see NEPM 2011, Schedule B2). Indeed, even when such taxonomic resolution is not required, the verification of identifications by a second party is desirable as part of QA/QC procedures.

In the absence of taxonomic expertise, molecular (genetic) approaches to taxonomy may be appropriate. In particular, the use of the cytochrome oxidase barcoding gene or the 18S ribosomal RNA gene is widespread for invertebrate identification, and is likely to be useful here. These analyses are increasingly available from commercial laboratories, and are likely to be relatively cost-effective in comparison to traditional taxonomic approaches. As a note of caution, the extraction and amplification of DNA from stygofauna can be difficult, with a low (30 – 50%) success rate. As a consequence, a large number of specimens may need to be analysed before sufficient data is returned.

Molecular approaches will not be able to provide a species name to a specimen, because most species collected will be undescribed and their genetic information not present in existing DNA databases. Molecular approaches, however, do allow for the identification and discrimination of different species within a study, and can confirm the higher level classifications of specimens by reference to genetic databases. WA EPA (2007) provides a useful discussion on the use of molecular-based taxonomy of groundwater fauna and the use of genetic divergence for species delineation.

3.5 Sampling methods for microbial assemblages

Unlike sampling for stygofauna, microbial sampling requires that the bore be purged, with existing water within a bore removed and replaced by water from the surrounding aquifer. In this way, there is a greater likelihood that microbial assemblages collected in water samples reflect the properties of the aquifer instead of the bore, which is likely to have been stagnant and influenced by exposure to the surface.

Purging requires the removal of 3 to 5 bore volumes, which parallels the requirements of pump sampling discussed above. The pump type used appears less critical for

microbial sampling, as microbes are sufficiently small enough to not be damaged by impellor pumps. However, risk of cross contamination between samples complicates sampling procedures, lending favour to pump mechanisms that can be easily and reliably sterilised. In this regard, inertia, air lift or peristaltic pumps (in which samples are restricted to pump tubing, and do not contact mechanical pump workings) appear popular. However, no formal comparison of pump sampling efficiencies for microbial sampling has been published.

Bailers can also be used to sample microbial communities, but may be limited to use in shallow, low-volume bores where it is feasible to purge the bore with this method. The decontamination of equipment in the field is often difficult, and so the use of sterile, disposable bailers makes sampling and management of contamination easier.

Contamination is a recurring issue in any microbiological sampling. Avoiding sample contamination is critical to reliable assessment of aquifer microbial assemblages. All surfaces in contact with samples (e.g. sampling equipment, sample containers) should be sterilised prior to sample collection, and equipment should be decontaminated before using it to collect further samples. Details of decontamination procedures, specifically for microbiological sampling, are provided in Sundaram et al. (2009).

Contamination of groundwater samples with surface water or soil should be avoided, and care must be taken to avoid dislodging soil, organic matter or other debris from the surface around the bore into the bore hole. This is particularly pertinent when sampling bores that are capped at ground level and do not have a casing that extends above ground; soil and vegetation around the bore and/or insects and spiders living within the bore housing can be easily dislodged to fall into the bore. Similarly, care must be taken to prevent water removed from the bore from running back into the bore hole. All water removed from a bore should be disposed of according to relevant regulations.

Microbiological samples should be placed on ice or refrigerated immediately after collection. They should be analysed as soon as possible, ideally within 24 h. A flow chart for microbiological sampling methods is provided in Appendix 2.

3.6 Processing samples for microbial assessment

The majority of groundwater microbes are sparsely dispersed as single cells or small colonies attached to sediment surfaces (Novarino et al 1997; Humphreys 2006). Generally, less than 1% of available sediment surfaces are colonised by bacteria (Griebler et al. 2002; Anneser et al. 2010); healthy, undisturbed aquifers tend to have very low microbial diversity and activity relative to surface waters (Griebler and Lueders 2009). It also appears that most microbes inhabiting aquifers are attached rather than being free-living (Gounot 1994; Griebler nd Lueders 2009; Anneser et al. 2010) – although the ratio of attached to free-living bacteria can change with contamination (e.g. Griebler et al. 2002).

Given the low abundance and diversity of microbes in groundwater, there is often a need to concentrate the microbes in samples. Filtration of water samples using 0.2 – 0.45 µm filters captures suspended sediment and associated microbes, and free-living microbes. Resuspension of filtered material into a small volume provides a concentrated microbial suspension for analysis. The approach used for sample processing will depend on the subsequent method of analysis.

4. Sampling strategies

4.1 Stygofauna sampling

Heterogeneity is perhaps the key defining attribute of groundwater stygofauna assemblages. This is probably due, in part, to the very small sampling area of groundwater sampling relative to the total three dimensional space of an aquifer, coupled with a limited ability to examine the relative representativeness of the sample collected. For example, it is quite easy when sampling a section of river habitat or vegetation to have some certainty that the area from which you are collecting represents that which is around you. In aquifers this is not readily possible.

The consequence of the heterogeneity of stygofauna distributions is that a high level of replicated sampling may be needed in order to assess the biodiversity of groundwater fauna. In sampling a range of aquifer types in the Pilbara, Western Australia, Eberhard et al. (2009) found that a large number of species collected had very narrow distributions, restricted to particular aquifers. Within bores sampled across the 178,000 km^2 Pilbara region, a high degree of endemism in the fauna – i.e. a single sample (using net sampling) collected 33% on average of the total taxa from a bore, while 6 sampling events (taken over several years) collected 82% on average. Species accumulation curves suggest that 10 sampling events may be necessary in order to adequately assess the stygofauna in some locations. The WA EPA (2007) guidelines suggest 12 samples are necessary to collect 95% of taxa. This may be done as 12 repeat samples from a single bore, 6 from 2 bores etc. Importantly, this work was done with a very high level of taxonomic resolution.

At a smaller spatial scale, Hancock and Boulton (2009) needed more than 4 samples (over consecutive seasons) to capture the full taxonomic richness of most bores in alluvial aquifers of NSW. A single pumped sample collected between 30 – 87% of the total taxa from each bore. Increasing taxonomic richness coincided with seasonal sampling, so it is unclear how much of the variability in composition of repeat samples is related to sampling error or seasonal changes. This study used a simpler approach to taxonomy than that of Eberhard et al. (2009), which is likely to have resulted in an underestimate of total richness (Hancock and Boulton 2009). Importantly, this paper highlights potential seasonal variation in faunal richness at the scale of the aquifer/ alluvial system (bores separated by up to approximately 15 km), and the need for multiple samples in order to adequately assess stygofauna assemblages.

At a similar scale, but with more frequent sampling, Hose and Lategan (in prep) found large variation in the trends of richness with sampling effort in the alluvium of Wollombi Brook, NSW. In the richest bore, 4 repeat samples (over a period of 7 weeks) were needed to collect all taxa from a single bore, but in bores with no or only 2 taxa collected in total, a single sample adequately reflected the stygofauna richness of that bore.

Sampling at finer spatial scales, with less than 300 m between bores and sampling at similar depths, both multiple spatial and temporal samples were required to assess site richness. In a coastal sand aquifer, at least 2 samples were needed to assess stygofauna richness in most bores (Hose and Lategan in prep).

However, in a fractured sandstone aquifer, 5 repeat sampling events over 15 weeks were needed to assess the richness of most bores, and the aquifer overall (pooled across all 5 bores; see Figure 3). Importantly, in some bores, no stygofauna were detected until the fourth sampling event, highlighting the need for spatial and temporal replication of sampling.
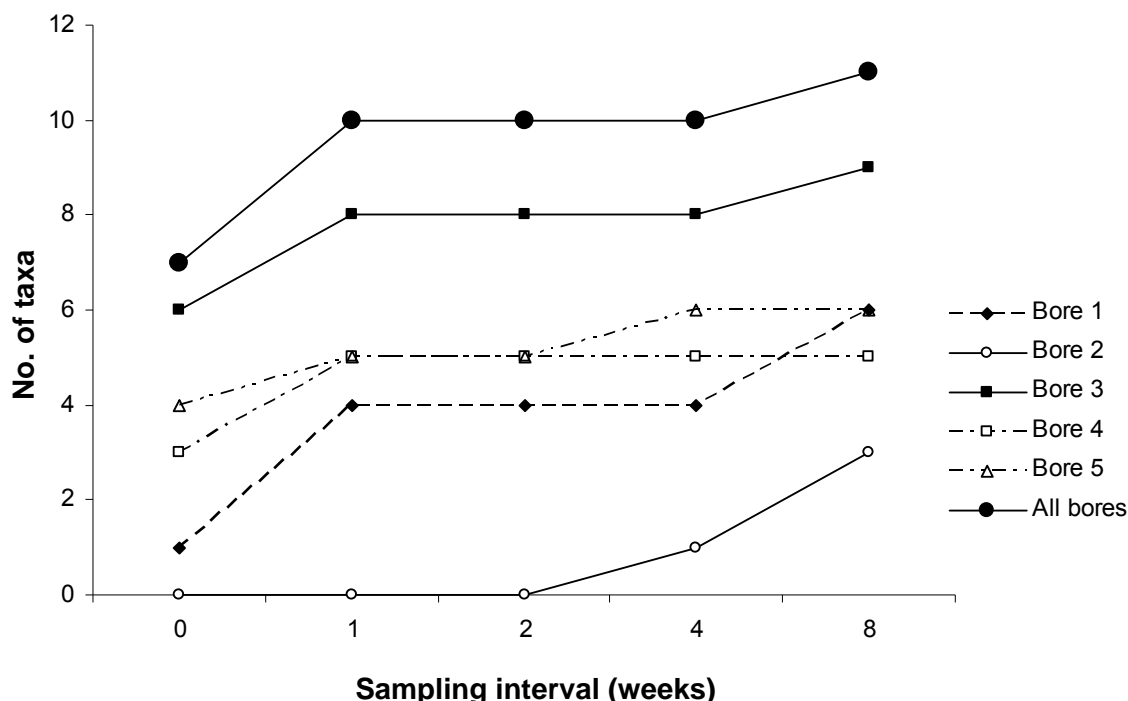
Figure 3. Repeat sampling of stygofauna from a fractured sandstone aquifer in NSW.

Note: With five bores sampled (and pooled), new taxa were still being collected on the fifth sampling event. Any single bore contained only a portion of the total richness found across the region.

Source: Hose & Lategan (unpub data).

The key messages for stygofauna sampling are thus:

- multiple sampling events are necessary to assess stygofauna assemblages in any bore

- multiple bores per aquifer/region are needed to assess the richness of that aquifer/region

- there is some evidence to suggest inter-seasonal variability in stygofauna assemblages, suggesting that sampling should cover this temporal range, and

- the variability in stygofauna sampling over small spatial scales appears to be of a similar magnitude to that occurring at seasonal (Hancock and Boulton 2009) and annual/biannual scales (Eberhard et al. 2009)

4.2 Microbial sampling

With a large number of potential endpoints for microbial analysis (see Goldscheider et al. 2006; Korbel and Hose 2011), it is likely that sampling strategies and designs for each will also vary.

Nevertheless, discussed here are a range of indicators covering broad microbial activity. Measures of specific microbial activity may only target a component of the microbial community which may not follow the trends of the more general indicators.

Sampling strategies for such measures should be considered and developed as needed. Similarly, molecular approaches were not trialled in this initial study, but these methods are increasingly being used for comparing microbial assemblages in aquifers. Analysis of 16S and 18S rDNA have been particularly useful for characterising prokaryotic and eukaryotic microbes in contaminated groundwater (e.g. Feris et al. 2004; Brad et al. 2008). Methods such as terminal restriction fragment polymorphisms (TRFLP) and single-stranded conformation polymorphisms (SSCP) provide little information on the identity of microbes in an assemblages, but are useful for comparing different microbial assemblages (e.g. Stein et al. 2010). Fluorescence in-situ hydribisation (FISH) can be used to identify specific functional genes, or to identify microbial assemblage composition in groundwater (e.g. Caracciolo et al. 2010). While cloning and sequencing is still widely used for identifying key microbial taxa, full pyrosequencing of microbial assemblages promises to greatly increase our knowledge of microbial diversity in groundwater systems.

The following discussion considers approaches for assessing microbial assemblages based on ex-situ assessment or culture of microbes. It is well-recognised that such approaches may only reflect a proportion of the total microbial assemblage. Those not represented by these methods being those that are either non-viable ex-situ, or are killed by virtue of being removed from the groundwater environment (e.g. obligate anaerobes).

Analysis of microbial assemblages in groundwater and their activity can be done in many ways, including molecular fingerprinting, metabolic fingerprinting, enzyme assays etc. Frequently, the composition of microbial assemblages is highly heterogeneous, and complicated by difficulties in identifying many environmental bacteria (Griebler and Leuders 2009). Consequently, assessments of microbial assemblages are best based on community level, rather than population level measures. As microbial assemblages are the key drivers in water purification in aquifers, it is often measures of microbial functional activity that are most important for monitoring and assessment.

In the following discussion of sampling strategies, microbial activity was assessed in three ways. The first was by metabolic fingerprinting using commercially available BIOLOG™ plates. The others are of general (FDA) and more specific (*B*-glucosidase) enzymatic activity.

Estimates of bacterial abundance can vary by several orders of magnitude over small spatial and temporal scales (Lategan unpub data), leading to this broad measure perhaps being unreliable in a monitoring or impact assessment context. Measures of microbial activity are likely to have greater relevance to issues of environmental change because they reflect the assemblage function and, in many cases, are directly related to the ecosystem services provided by groundwater ecosystems (see Boulton et al. 2008). Flourescein diacetate hydrolysis by microbial assemblages has been used widely as an indicator of microbial activity in soil and hyporheic environments (e.g. Boulton and Quinn 2000) and, to a lesser extent, groundwater ecosystems.

Across a number of aquifers of varying geology (e.g. alluvial, coastal sands, fractured sandstone), rates of flourescein diacetate hydrolysis by microbes varied markedly between bores and over time. However, the level of variation among relatively undisturbed sites appears smaller than that observed (Lategan unpub data) between undisturbed and disturbed (at least organically enriched) sites (e.g. Federle et al. 1990, see Figure 4), suggesting that measuring general microbial activity via the hydrolysis of FDA may be a useful tool for groundwater ecosystem assessment and monitoring. Importantly, the variability among even closely located bores necessitates that samples are collected over space and time. While the level of general microbial activity varied among samples, it did not do so in a repeatable pattern, such that inter-sampling intervals greater than 1 week appear to have little influence on sampling results.
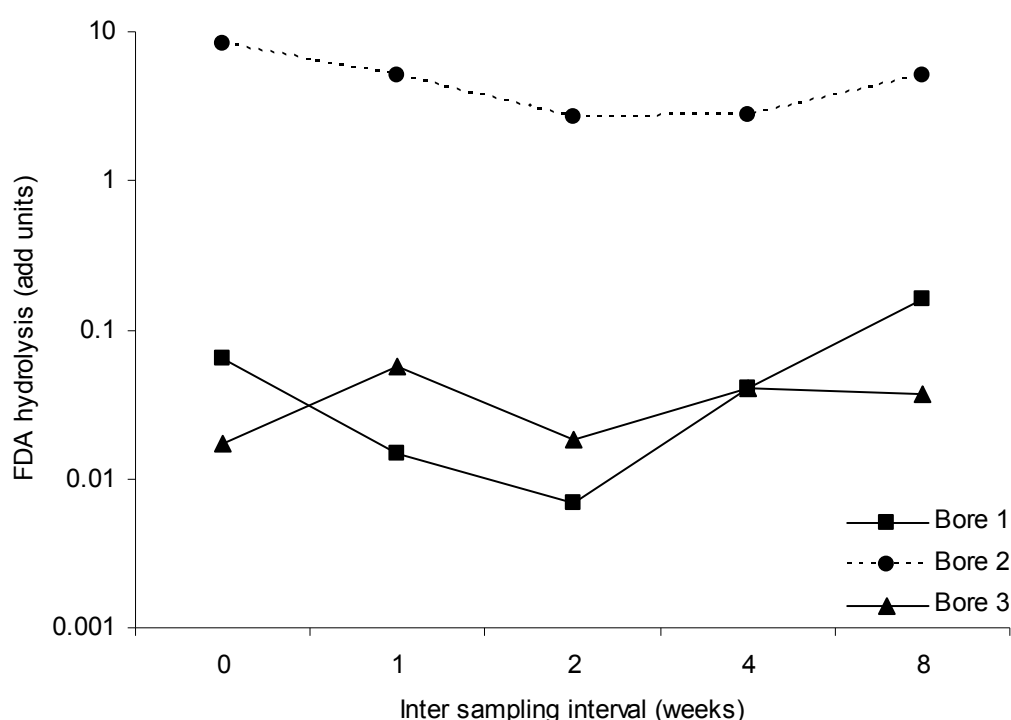
Figure 4. Temporal variability in fluorescein diacetate (FDA) hydrolysis rates was evident in reference sites (bores 1 and 2) in a coastal sand aquifer.

Note: Temporal variation was of a similar magnitude to spatial variation among the reference sites. The reference sites alone show variation over one order of magnitude, but relative to the FDA rates in disturbed locations, the variation in reference sites is acceptable and potentially useful for detecting significant changes in microbial FDA activity.

Source: Lategan & Hose (unpub).

Microbial metabolic fingerprinting relies on the preferential use of particular carbon sources by microbes as an indicator of differences in assemblage structure. There are several types of commercially available, ready-to-use plates, such as the BIOLOG™ Ecoplate. The BIOLOG™ Ecoplate has been used to infer change in microbial assemblages in a range of environments, including groundwaters (e.g. Röling et al. 2000).

It is useful to employ and inoculate duplicate plates for a particular sample because of the potential for loss of replicates (e.g. by natural, quick-growing fungal elements, which can obscure optical measurements, Lategan pers. obs.). However, the repeatability of duplicate samples using Ecoplates is good (Lategan unpub data). Temporal and spatial variability in microbial physiological profiles was evident across different aquifer types. In different aquifers, spatial or temporal patterns dominated.

For example, when repeatedly sampling bores that were separated by less than 100 m in a coastal sand aquifer, the resulting samples showed greater fidelity to bores (location) rather than to times, suggesting spatial patterns dominating over temporal patterns. Conversely, closely located bores in other aquifers showed greater variability over time than to space (Lategan unpub data). In terms of using BIOLOG™ Ecoplates for monitoring and impact assessment, temporal and spatial replication must be included in the study design.

The key messages for microbial sampling are thus:

- microbial assessments involving ex-situ methods of analysis may only reflect a proportion of the groundwater microbial assemblage

- the level of sample replication required will depend on the analytical approach and the variables measured

- multiple bores per aquifer/region are needed to assess the microbial activity of that aquifer/region, and

- microbial assemblages and microbial activity can vary greatly over space and time. The nature of such variability should be understood prior to implementing a monitoring or impact assessment program.

5. Sample handling, labelling and documentation

The possession and handling of samples should be traceable and documented from the time of collection through a 'chain of custody'. Components of the chain of custody and procedures for their use are described in AWRC (1991) for environmental sampling. An example chain of custody record form is provided in Appendix 3.

All samples should be labeled with a unique, identifiable sample code that can be traced via chain of custody and related back to the field sampling data sheet. All samples should be labeled clearly with a unique sample code. Stygofauna sample labels should be written in pencil on waterproof paper and stored with the sample inside the sample container. Samples for microbial analysis should be labeled externally to avoid contamination of the sample. In addition to the sample code, sample labels should include at least:

- the project name or number
- name or initials of the person who collected the sample
- the date and time the sample was collected
- the location from which the sample was collected, and
- the method of preservation used (e.g. ethanol, formaldehyde, non-preserved etc.).

The unique sample code should be traceable to the field data sheet, on which details of the sampling method and conditions at the time of sampling are recorded. The field data sheet should include details of the bore location and construction, details of the sampling approach (such as sampling method (pump, bailer, net etc.) and pump rate), physico-chemical water quality (as an indication of bore purging and environmental conditions), standing water level, and aspects of quality control and quality assurance. As bores can be difficult to (re)locate, a detailed description of bore location is also useful. An example field data sheet is provided in Appendix D.

6. Conclusion

Groundwater ecosystems differ markedly from surface systems in terms of their biota and ecological processes. Accordingly, sampling and assessment of groundwater biota for the purpose of environmental monitoring and assessment will require specific methodologies and sampling strategies. Both microbial and macro-(meio)invertebrate fauna should be analysed to provide a comprehensive ecosystem assessment. However, both microbial and macro-(meio)invertebrate assemblages in groundwater display considerable spatial and temporal heterogeneity, requiring spatially and temporally replicated sampling to achieve both adequate representation of biodiversity, and to permit inference-based testing in an impact assessment or monitoring framework. The nature of the sampling variability among space and time appears to vary considerably among aquifers and over time, requiring some preliminary site-specific investigations.

7. Acknowledgements

8. References

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APPENDIX A.

Flowchart for stygofauna sampling of groundwater

```
                    ┌─────────────────────────────────────────┐
                    │   Select stygofauna sampling method     │
                    └─────────────────────────────────────────┘
```

Pump | **Net** | **Bailer**

Pump	Net	Bailer
Ensure prior decontamination of sampling equipment. Sterilise inside and outside of tubing. Ensure sieve is rinsed to remove any material from previous samples.	Ensure prior decontamination of sampling equipment. Rinse net thoroughly to remove any material from previous samples.	Ensure prior decontamination of sampling equipment. Sterilise inside and outside of the bailer. In doing so, remove any material from previous samples.
Where possible pump 300 L of water. If 300 L cannot be collected, document volume retrieved.	Complete 3 hauls with fine 100-150*-µm mesh net. Empty net and retain contents after each haul.	Drop bailer into bore and retrieve. Empty bailer and retain contents after each haul. Repeat until desired volume of water removed.
Regularly measure physico-chemical water quality and check groundwater drawdown.	Complete 3 hauls with coarse 50*-63 µm mesh net. Empty net and retain contents after each haul.	Combine contents from each of the hauls and record total volume collected. Sieve sample (50*-63 µm mesh) to facilitate sample preservation, storage and transport
Sieve water collected (50-63 µm mesh) and retain sieve contents.	Combine net contents from the 6 hauls and sieve sample (50*-63 µm mesh) to facilitate sample preservation, storage and transport.	

```
┌─────────────────────────────────────────────────────────────────┐
│ Label and preserve sample for laboratory processing. Complete    │
│ Chain of Custody documentation and field data sheet.             │
└─────────────────────────────────────────────────────────────────┘

┌─────────────────────────────────────────────────────────────────┐
│ Decontaminate sampling equipment ready for next use.             │
└─────────────────────────────────────────────────────────────────┘
```

APPENDIX B.

Flowchart for mocrobiological sampling of groundwater

```
┌─────────────────────────────────────────────┐
│       Select microbial sampling method        │
└─────────────────────────────────────────────┘
        │                              │
        ▼                              ▼
   ┌─────────┐                    ┌─────────┐
   │  Pump   │                    │ Bailer  │
   └─────────┘                    └─────────┘
        │                              │
        └──────────────┬───────────────┘
                       ▼
┌─────────────────────────────────────────────┐
│  Ensure prior decontamination of sampling     │
│  equipment and sterility of sample            │
│  collection vessel.                           │
└─────────────────────────────────────────────┘
                       │
                       ▼
┌─────────────────────────────────────────────┐
│  Remove 3-5 bore volumes of water to purge    │
│  bore before collection of a sample.          │
└─────────────────────────────────────────────┘
                       │
                       ▼
┌─────────────────────────────────────────────┐
│  Measure physico-chemical water quality       │
│  prior to sample collection.                  │
└─────────────────────────────────────────────┘
                       │
                       ▼
┌─────────────────────────────────────────────┐
│  Collect water sample in a labeled, sterile   │
│  container.                                   │
│  Store sample immediately on ice or           │
│  refrigerate for transport.                   │
│  Analyse/process within 24 h.                 │
└─────────────────────────────────────────────┘
                       │
                       ▼
┌─────────────────────────────────────────────┐
│  Complete Chain of Custody documentation and  │
│  field data sheet.                            │
└─────────────────────────────────────────────┘
```

APPENDIX C.

Example chain of custody form (adapted from SA EPA 2007)

Sample Collector		Laboratory	
Project name		Contact name	
Contact name		Address	
Tel:		Tel:	

Sample code	Container	Collection date	Collection time	Sample type (stygofauna, water chem, microbiological)	Container volume (mL)	Preservative added (give detail eg ethanol, formalin)	Storage requirements (e.g. 4°)	Analyses required	Notes
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			
	__ of __					Y/N			

Sample relinquished by:				Sample received by				
Name/organisation	Initials	Date	Time	Name/organisation	signature	Date	Time	Sample condition

Sampling strategies for biological assessment of groundwater ecosystems

APPENDIX D.

Example field data sheet for groundwater biological sampling

Field Data Sheet

Logger out:_____(time)
Logger in:_____

Sample code: _____

Project: _____

Date : _____

Location: _____

Sampling team: _____

Bore No: _____

Total bore depth (inc case): _____m   Standing Water level (inc case): _____m Slot depth (inc case): _____m

Case height: _____m   Bore Volume: _____L   Bore diam: _____mm
(50 mm bore = 1.96 x depth m)
(100 mm bore = 7.86 x depth m)

Site Sketch

Site details & Access

Lat: _____ Long: _____

_____

_____

_____

_____

_____

_____

_____

_____

Stygofauna samples

Net sample collected: Yes/No
No Hauls: ____   Mesh size: _____   ☐ Sample separate   ☐ Combined w pump sample
No Hauls: ____   Mesh size: _____

Pump sample collected: Yes/No
Volume: _____ L Sieve Mesh: _____   Sample ☐ Live ☐ Preserved   No. Jars:_____

Microbiological samples

Vol pumped before sample taken: _____ L Vol. of sample collected: ____ L Sample on ice: Yes/No

Water Quality

Volume pumped					
DO					
pH					
Cond					
Temp					
Alkalinity					
ORP					

Pump rate estimate (volume/time)

Volume pumped _____ L
Time taken: min   sec

QA/QC
☐ WQ meters calibrated
☐ Pump tube cleaned after
☐ Sieve cleaned after
☐ Chain of custody form

Comments_____

_____

_____