

## Physico-chemical profile and energy fixation in Bundera Sinkhole, an anchialine remiped habitat in north-western Australia

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### Abstract

Bundera Sinkhole, in arid north-western Australia, contains a community of stygofauna, including remipedes and thaumatocypridid ostracods, whose closest known affinities lie with the fauna of anchialine caves on either side of the North Atlantic. The physico-chemical profile was determined using a sonde carried by divers and by chemical analysis. The physico-chemical profile is complex, as the characteristic thermo-halocline is associated with polymodal profiles of oxygen, hydrogen sulphide and redox to a depth of 33 m. Within the upper part of the profile there was a gradient in the dissolved inorganic nitrogen (DIN) species associated with the thermo-halocline, and an accumulation of  $\text{NO}_3^-$  below the thermo-halocline that is consistent with nitrification, a chemolithotrophic process. The presence of sulphide layers above the thermo-halocline, together with characteristic colour and behaviour of the associated bacteria, are consistent with sulphide-based chemoautotrophy. Stable isotope ratios for carbon and nitrogen of the fauna and autotrophic energy sources are also consistent with chemosynthesis occurring in Bundera Sinkhole. The arid climate, low ground-water flow, and small size of the cave provides the stable conditions allowing the development of a marked polymodal vertical stratification in the physico-chemical profile. The input of profuse organic matter and the penetration of the photic zone to the thermo-halocline provide the varied conditions for the development of this complex microbiological assemblage.

### Introduction

Anchialine (or anchihaline) ecosystems are inland underground mixohaline waters affected by marine tides, usually with little if any surface exposure (Stock *et al.* 1986; Iliffe in press). Anchialine systems are noted both for their relict faunas and their high species richness (Sket 1981, 1996); at least ten new families and a new class (the Remipedia) of crustaceans have been described from anchialine systems in the last few years (GA Boxshall, Natural History Museum, London, pers. comm.). Therefore they are the object of an accelerating research effort and widespread conservation assessment as their restricted, coastal warm latitude, often karstic, setting makes them exceptionally vulnerable to anthropogenic pressures, a theme developed more fully elsewhere (Humphreys *et al.* 1999).

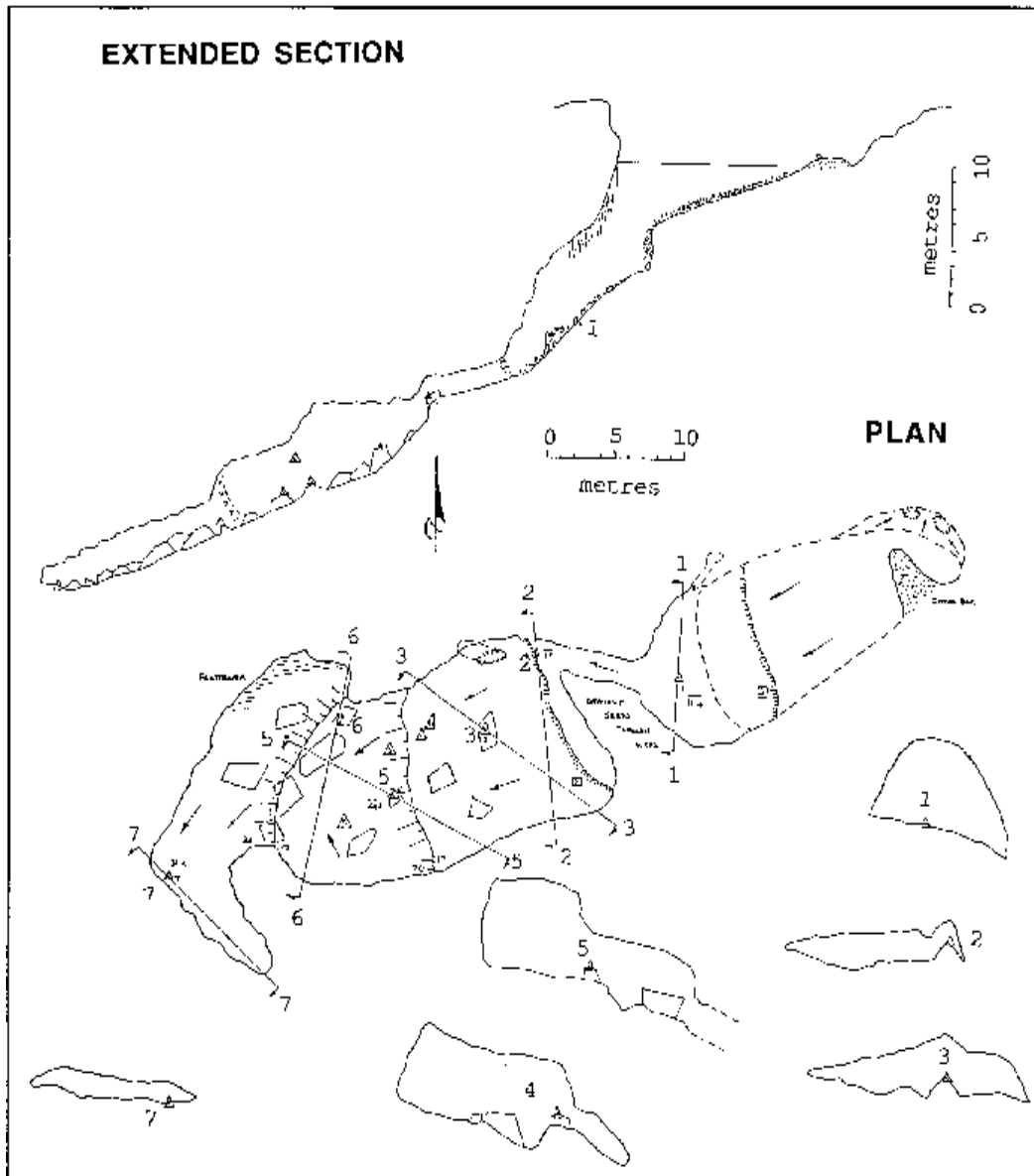
Where anchialine systems occur in drowned sinkholes (cenotes), they typically have a freshwater layer overlying seawater and in consequence have a stratified photic and physico-chemical profile. The marine influence typically consists of an hypoxic layer of seawater (ca. 33-36 g L<sup>-1</sup>) beneath one or more layers of limnetic to polyhaline water (Yager *et al.* 1994). Below the density interface, such ecosystems support a wide range of relict life forms that comprise a community whose general structure is predictable from the Caribbean region, the Canary Islands and north-western Australia (e.g. Baltanas & Danielopol 1995; Yager 1981, 1987a,b, 1989, 1994; Yager & Schram 1986; Holsinger 1989; Danielopol 1990; Yager *et al.*

1994; Yager & Humphreys 1996; Danielopol *et al.* in press).

Remipedes have always been collected beneath a density interface (thermo-halocline) in hypoxic water of 0.1-0.22 mg L<sup>-1</sup> DO, sometimes beneath a layer of hydrogen sulphide (Yager 1994; Yager *et al.* 1994). The integrity of such anchialine ecosystems may depend on the maintenance of this stratification. Filamentous sulphur bacteria of the *Beggiatoa-Thiothrix* group can be common in anchialine caves and their hypothesised contribution to primary production by chemoautotrophy (Yager 1991a; Yager & Humphreys 1996) has been supported using stable isotope analyses (SIA: Pohlman *et al.* 1997).

The only deep anchialine system known in Australia, and the only continental anchialine system known in the southern hemisphere (see Iliffe in press: Fig 1), is Bundera Sinkhole in north-western Australia. This ecosystem includes the only known site for the crustacean class Remipedia in the southern hemisphere (Yager & Humphreys 1996), in an area containing the characteristic higher taxa of stygobiont crustaceans comprising thermosbaenaceans, hadziid amphipods, ostracods, cirolanid isopods, mysids, and caridean shrimp (Humphreys in press). In this arid area the surface layers are not fresh but have a salinity about half seawater rising to seawater at depth (Yager & Humphreys 1996).

This study documents the gross physico-chemical profile of the Bundera Sinkhole (temperature, salinity, dissolved oxygen, pH, redox), and the fine scale distribution at selected depths of H<sub>2</sub>S and dissolved



**Figure 1.** Plan and extended section of Bundera Sinkhole. The floor is mainly boulder breakdown. Algal/bacterial growth is depicted by the fringes near the top of the section. The lines vvvv denote the limit of ambient daylight and the limits of daylight sighting under optimal (first-dive) conditions. The numbered lines in the plan denote the location of the cross sections below numbered 1 to 7. Surveyed by A Poole and D Warren, 24-26 May 1995 to Grade BCRA Standard 3C.

inorganic nitrogen species (nitrite, nitrate and ammonium), and the stable isotope signatures ( $^{13}\text{C}$  and  $^{15}\text{N}$ ) of various components.

## Methods

### Bundera Sinkhole

The flooded sinkhole is located on the Cape Range peninsula in north-western Australia and is situated 1.7 km inland from the Indian Ocean. The cave has a single entrance from which a flooded passage, inclined at ca.  $30^\circ$  from the horizontal, extends about 70 m (Fig 1) to a maximum depth penetrated by divers of 33 m. The sinkhole is situated in the middle of a flat, 4.5 km wide coastal plain and the water table lies ca. 5 m below the surrounding surface. The depth to water and the small

area of the sinkhole (ca. 10 x 20 m) will contribute to the stability of the water profile by shielding it from the often strong winds.

Characteristic of anchialine systems, the water level is influenced by the semi-diurnal marine tides of the area and which have an amplitude ca. 16% of the ocean tides (Humphreys *et al.* 1999). The tidal influence also affects the sinkhole in other ways; at high water the algal mats are submerged and the water surface is clear, but at low water the algae are emergent and, by spreading over the surface, greatly restrict light penetration.

A number of obligate subterranean species occur in the sinkhole below the thermo-halocline and are known only from this site. They include *Lasioneetes exleyi* Yager & Humphreys (Remipedia), *Danielopolina* sp nov Danielopol *et al.* in press (Ostracoda: Halocyprida:

Thaumatoctyprididae), *Liagoceradocus branchialis* Bradbury & Williams (Crustacea: Hadziidae), as well as the more widespread species *Stygiocaris stylifera* Holthuis (Decapoda: Natantia: Atyidae) and *Milyeringa veritas* Whitely (Perciformes: Eleotridae). Above the thermo-halocline are found the algae *Rhizoclonium ?tortuosum* (Dillw) Kuetz (Chlorophyta: Cladophoraceae) and *Lamprothamnium papulosum* (Wallr) J Gr (Charophyta: Characeae), and the invertebrates ?Spionidae (Annelida: Polychaeta), *Halicyclops* sp nov (Copepoda: Cyclopidae), *Phlyctenophora* sp Brady (Ostracoda: Cypridacea), *Iravadia* sp (Mollusca: Iravadiidae), water striders *Limnogonus* (L) *fossarum gilguy* Andersen & Weir (Gerridae: Hemiptera) and *Kiefferulus intertinctus* Skuse (Chironomidae, Diptera).

### Climate

The climate of the Cape Range peninsula is hot (mean daily temperature of ca. 27 °C) and arid and the annual evaporation (3219 mm) greatly exceeds the sparse precipitation (median 280 mm). Vegetation in the immediate vicinity of the sinkhole comprises predominantly hummock grassland and occasional shrubs.

### Diving

The cave is thought to have been dived on only six sessions covering the years 1991 to 1998. Diving has mostly been conducted using SCUBA gear typical of that used for cave diving with built in redundancy. The current samples were collected using rebreathing gear (Dräger Atlantis) to minimise the impact of diving on the water column (Humphreys *et al.* 1999), known to be highly stratified (Yager & Humphreys 1996; WF Humphreys unpubl. obs.).

### Sonde

Vertical profiles of depth (m), temperature (°C), dissolved oxygen (DO, mg L<sup>-1</sup>), redox (mV), pH, and salinity (mg L<sup>-1</sup> TDS) were made using a 44 mm diameter sonde (Datasonde3 Multiparameter Water Quality Datalogger DS3, Hydrolab Corporation, Austin, Texas) in untethered mode. During the first dive the sonde was pushed (sensors foremost) by the lead diver to record the undisturbed water column and sampling every second. The probes were calibrated against appropriate standards traceable to international standards. A LoFlow membrane was used on the DO probe without a stirrer. DO readings were calibrated to a salinity (TDS) of 18 g L<sup>-1</sup> and not further corrected. Redox values were measured with a silver-silver chloride reference electrode and the readings offset to be based on the standard hydrogen electrode using the European convention (reducing environment negative). The data were transferred to a computer at the end of the dive.

### Sample collection

Debris, sediments, animals and plants were collected from the immediate environment and at various depth in the sinkhole. Divers collected fauna and organic detritus from the sinkhole using towed plankton nets and hand-held suction devices, nets and bottles. Sediment and organic samples were collected into vials or polyethylene bags for transport to the surface. Terrestrial vegetation samples were stored in polyethylene bags. Organic

samples for SIA analysis were frozen until dried in an oven at 45 °C and ground under liquid nitrogen. Chemical analysis was conducted on water samples collected through a range of depths determined from the redox and DO profiles measured during the first dive. The water samples were collected using a 50 mL syringe at predetermined depths measured with a gauge.

### Chemical analyses

Total acid soluble sulphides (as mg L<sup>-1</sup> S; referred to as H<sub>2</sub>S in the text) were determined on site using a Chemetrics test kit (CHEMets sulphide R-9510, range 0-1 and 1-10 ppm). Nitrite, nitrate and ammonium nitrogen were determined within seven days by an analytical laboratory (Chemistry Centre, Perth) of samples frozen in the field. The standard methods used (Anon 1995) were: nitrogen, ammonium fraction 4500-NH<sub>4</sub><sup>+</sup> G automated phenate method; nitrogen, nitrite fraction, 4500-NO<sub>2</sub><sup>-</sup> B colorimetric method; nitrogen, nitrite + nitrate fraction, 4500-NO<sub>3</sub><sup>-</sup> F automated cadmium reduction method. Throughout square parentheses are used to denote concentration *e.g.* [H<sub>2</sub>S] is hydrogen sulphide concentration.

### Stable isotope analysis

Organismal metabolism utilizes the isotopes of an element differentially. Such 'fractionation' of the isotopes of nitrogen (<sup>15</sup>N/<sup>14</sup>N) and carbon (<sup>13</sup>C/<sup>12</sup>C) occurs in predictable ways such that consumers are typically enriched (are 'heavier') by about 3-5 ‰ in <sup>15</sup>N and 1-2 ‰ in <sup>13</sup>C relative to their food source (Ehleringer *et al.* 1986). Hence, stable isotope analysis (SIA) of nitrogen and carbon is a powerful tool to examine food webs (Peterson & Fry 1987; Lajtha & Michener 1994) and technical advances have recently allowed it to become a routine method in ecosystem and other studies.

Ecological studies express the isotopic composition in terms of δ values which are the parts per thousand differences from a standard:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3,$$

where X is <sup>13</sup>C or <sup>15</sup>N and R is the corresponding ratio <sup>13</sup>C/<sup>12</sup>C or <sup>15</sup>N/<sup>14</sup>N and the standards respectively are the Pee Dee Belemnite and nitrogen gas in the atmosphere (Peterson & Fry 1987).

SIA was conducted on plants and animals sampled both above the thermo-halocline (mollusc *Iravadia* sp, blind cave fish *Milyeringa*, gerrids and tubificid worm, and in the deep cave below the thermo-halocline and layers of hydrogen sulphide (atyd shrimp *Stygiocaris*, *Milyeringa*) and the algae *Rhizoclonium ?tortuosum* (Dillw) Kuetz (Chlorophyta: Cladophoraceae) and *Lamprothamnium papulosum* (Wallr) J Gr (Charophyta: Characeae). In addition, plants sampled from the immediate area included *Acacia phyllodes* and the hummock grass, *Triodia pungens*.

Total carbon and nitrogen and the fractionation of <sup>15</sup>N/<sup>14</sup>N and <sup>13</sup>C/<sup>12</sup>C determinations were made by the stable isotope facility, Centre for Catchment and In-Stream Research, Griffith University, Queensland, using an automated Dumas combustion elemental analyser ('Roboprep', Europa Scientific, Crewe, UK) interfaced directly with a triple collector mass spectrometer ('Tracermass', Europa Scientific). The precision of the

mass spectrometer, derived from the standard deviation of results of a replicated series of standards within a given sample run, are 0.5 delta units for nitrogen and 0.3 delta units for carbon (pers. comm., M Sutton, Griffith University).

## Results and Discussion

Here the physicochemical profile of the water in the sinkhole is described together with aspects of the associated microbiological community. The low oxygen levels and the stable isotope signatures of the biota are discussed as to the evidence they provide supporting chemoautotrophic energy fixation in the sinkhole. Finally the characteristics of Bundera Sinkhole are considered in relation to comparable systems elsewhere.

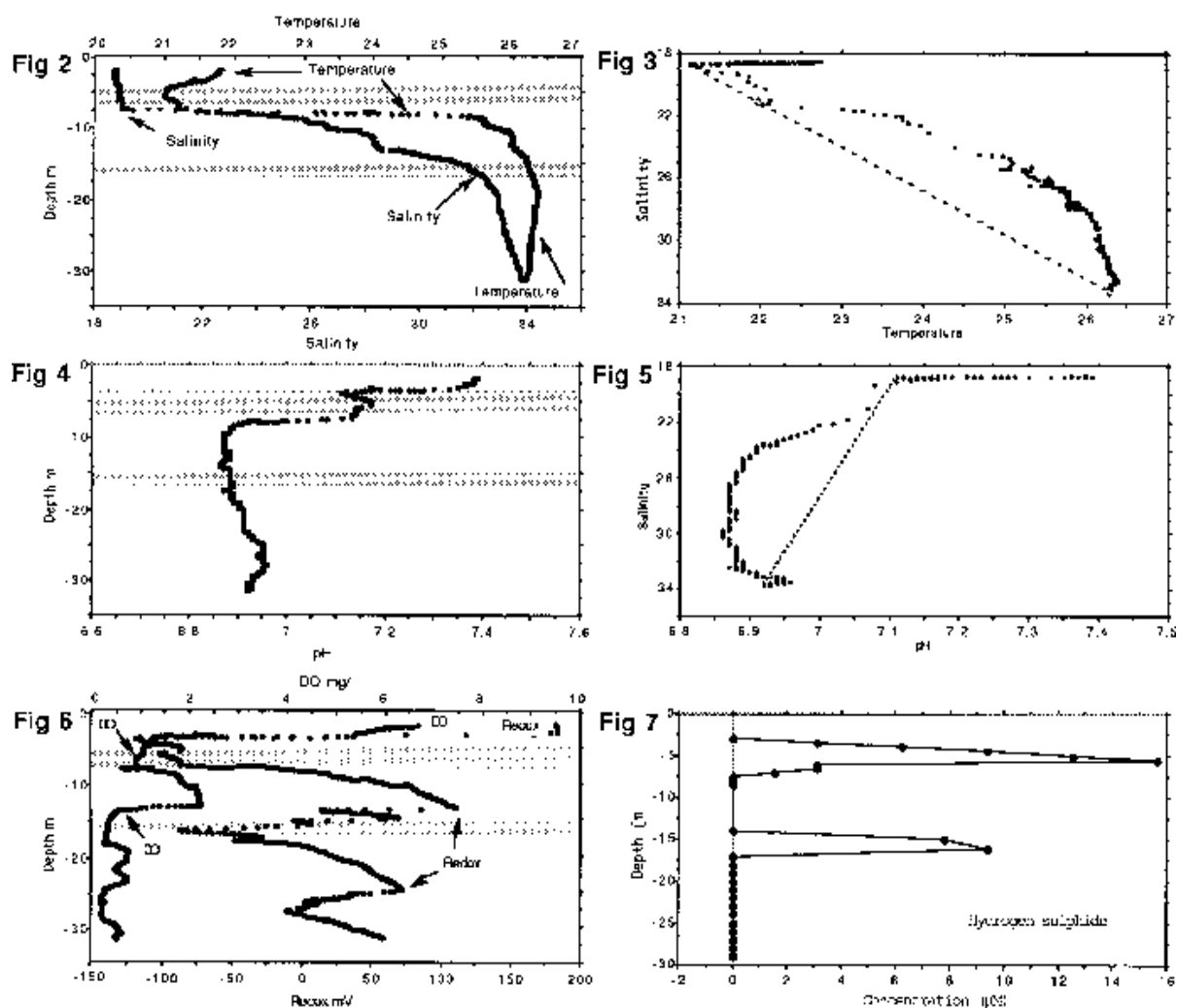
### Physico-chemical profile

The water column exhibited strong physico-chemical stratification with the different parameters exhibiting different types of stratification.

**Temperature and salinity.** There was a sharp thermocline at ca. 7.5 m where the temperature increased from about 21.0 to 26.5 °C, remaining close to this temperature to the depth of the cave (Fig 2A). The upper part of the profile can be in direct sunlight as it lies within the open part of the sinkhole (Fig 1). Under conditions of optimal visibility ambient day light penetrates to a depth of ca. 15 m and the entrance can be seen from a depth of ca. 25 m (Fig 1).

Surface salinity was ca. 19 g L<sup>-1</sup> down to a marked halocline at 7.5 m (Fig 2A), below which the salinity increased to ca. 24 g L<sup>-1</sup> and from which level salinity increased steadily to 32 g L<sup>-1</sup> at a depth of 15 m. The salinity then remain relatively constant increasing to 34 g L<sup>-1</sup> at a depth of 33 m (local seawater is 36.5 g L<sup>-1</sup>).

As expected, the deep temperature of ca. 26 °C is close to the mean annual surface temperature of the area (ca. 27 °C on the east coast) and it appears stable between years (Humphreys *et al.* 1999). The substantially cooler surface water probably results from evaporation from the exposed surface into the dry atmosphere. While thermal



**Figure 2.** Profiles of Bundera Sinkhole in 1997 where the grey bands denote the zones of elevated hydrogen sulphide concentration (see Fig 3F), the descent rate was 0.05 m s<sup>-1</sup> (sd 0.028, n=21). A; Temperature (°C) and salinity (g L<sup>-1</sup>TDS) profiles. B; the salinity-temperature relationship of the vertical profiles where the dotted line denotes the conservative mixing hypothesis. C; pH profile. D; the salinity-pH relationship of the vertical profiles where the dotted line denotes the conservative mixing hypothesis. E; dissolved oxygen (mg L<sup>-1</sup> DO) and oxidation reduction (redox) potential (mV) profiles. F; the vertical distribution of H<sub>2</sub>S (as total acid soluble sulphides, μM).

exchange with the surface is continuous, the sparse freshwater input at the surface is spasmodic and unpredictable (Humphreys *et al.* 1989) hence there is ample time for a broad zone of mixing (Fig 2A) to develop between the lower sea water and upper brackish water increasing in salinity with time owing to evaporation from the surface. The non-conservative mixing (Fig 2B) between temperature and salinity indicates that the water had warmed since entering the ground-water.

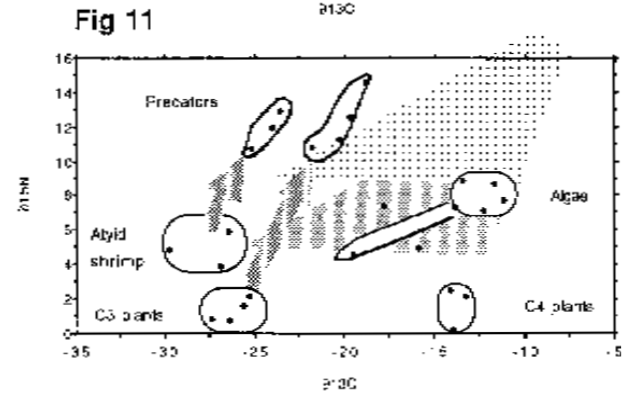
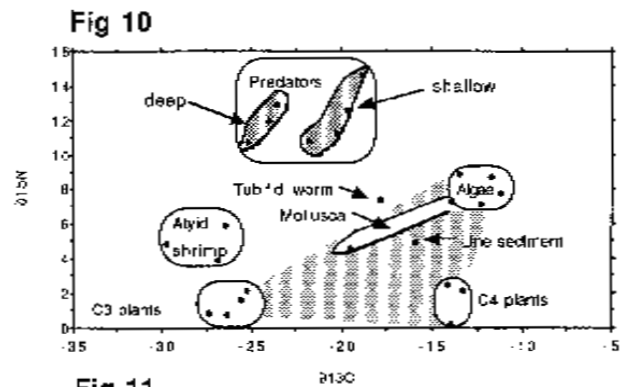
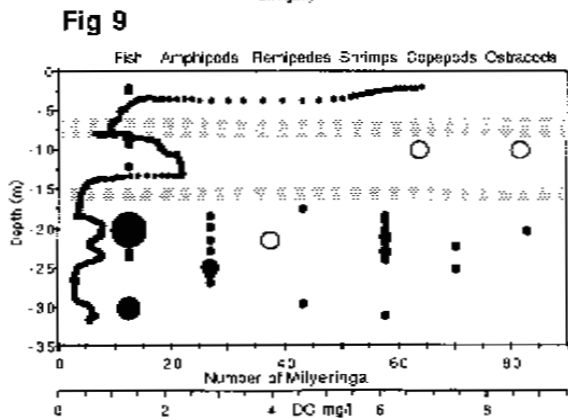
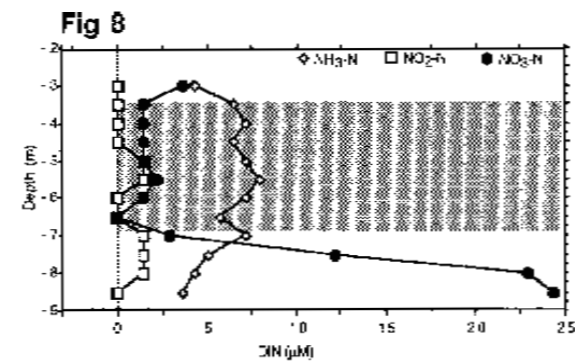
Temperature and salinity, the parameters most commonly measured to examine the physico-chemical profiles of water, had a fairly simple profile with an inverted thermocline but the salinity and temperature profiles were not concordant, with the halocline being much broader than the thermocline (Fig 2A). However, together these parameters provide a markedly oversimplified picture of the complexity of the vertical stratification present in Bundera Sinkhole.

**Hydrogen ion concentration.** pH declined from greater than 7.5 at the surface to ca. 6.9 at ca. 8 m at the bottom of the thermocline and then remains between ca. 6.9-7.0 to the bottom of the water column (Fig 2C). Below the

thermo-halocline the pH was fairly constant (6.90-6.96; Fig 2D). Non-conservative mixing is revealed by plotting pH against salinity (Fig 5) and due to hydrogen ion production at the halocline. This pattern is similar to that reported for the Mayan Blue Cave system, Mexico (Pohlman *et al.* 1997), but the details are different possibly due to the high photosynthetic activity in the surface waters of the sinkhole.

**Dissolved oxygen and redox.** DO fell rapidly from supersaturated at the surface to a minimum at about 7.5 m depth followed by additional minima at ca. 17 m and 27 m depth (Fig 2E). The complex DO curve is associated with the zones of increased  $[H_2S]$  although they are not entirely concordant. Stratification in the redox potential (Eh) was markedly polyphasic ranging from -125 mV to +175 mV. The redox minima correlated with oxygen minima but did not exactly map them; while each oxygen minimum occurred through a broad depth range (ca. 5 m), a redox minimum occupied only a narrow depth range in depth (Fig 2E).

**Hydrogen sulphide.** Two layers of elevated hydrogen sulphide, also detected by divers, were recorded at ca. 5 m and 16 m (Fig 2F) loosely concordant with the upper two



**Figure 3.** A; Vertical profile with depth of the dissolved inorganic nitrogen species (DIN  $\mu$ M)  $N-NH_4$ ,  $N-NO_2$ ,  $N-NO_3$ , shading denotes the region of elevated  $H_2S$  concentration (see Fig 2F). B; vertical distribution of sightings of the fauna in Bundera Sinkhole reported by divers, a) the closed circles denote the vertical distribution of the taxon named at the head of the column, b) the open circles denote the number of blind gudgeons (*Milyeringa veritas*) counted at one occasion at each of three depths (second bottom scale), c) the curve denotes the DO concentration (bottom scale), d) the grey bands denote the zones of elevated hydrogen sulphide concentration (see figure 9). These data are illustrative only as no regular sampling has been conducted through time. C; scatter plot of  $^{13}C$  ‰ and  $^{15}N$  ‰ values for samples from Bundera Sinkhole. If the food source is hypothesised to be some mix of  $C_3$  and  $C_4$  plants and algae then the average  $^{13}C$  and  $^{15}N$  values would lie in the polygon linking these data (shaded area). Data for plants with  $C_3$  and  $C_4$  metabolism, and algae are outlined, as are predators (left group, deep cave *Milyeringa*; right group, shallow cave *Milyeringa* [x3], gerrid [x1]), molluscs and worms. D; scatter plot of  $^{13}C$  ‰ and  $^{15}N$  ‰ values for samples from Bundera Sinkhole. If the food source is hypothesised to be some mix of  $C_3$  and  $C_4$  plants and algae then the average  $^{13}C$  and  $^{15}N$  values would lie in the polygon linking these data. This is represented as a shaded area but it has been displaced by one (heavy shading) and two (light shading) trophic increments of respectively 2 ‰ for  $^{13}C$  and 4 ‰ for  $^{15}N$ .

DO and redox minima (Figs 2F, 3A). Owing to the sampling resolution at depth in the sinkhole an additional layer of elevated  $H_2S$  associated with the deepest redox minimum may have been missed. In sediments, also, there can be a similar close correlation between the redox and  $S^{2-}$ -ion activity (Fenchel & Finlay 1995:11). This result at Bundera contrasts with the finding of Pohlman *et al.* (1997, in press) who recorded no dissolved sulphides ( $<2 \mu M$ ) in the water column of an anchialine cave.

The maximum recorded  $H_2S$  was  $15.6 \mu M$ . This compares with  $250 \mu M H_2S$  in freshwater of Movile cave (Sarbu in press; Table 1), the most notable sulphide driven subterranean ecosystem, but other chemoautotrophic sulphide caves systems are being recognized (Sarbu *et al.* in press; Pain 1998).

**Dissolved inorganic nitrogen species.** Large changes in the vertical distribution of the DIN species are associated with the thermocline (Fig 3A).  $N-NH_3$  is elevated in the upper  $H_2S$  layer that lay on the thermocline, while  $N-NO_3$  increases markedly at the thermocline. It is not known whether a similar stratification of the DIN species occurs associated with the deeper hydrogen sulphide layer (Fig 2F), or the even deeper redox minimum (Fig 2E). The upper layer of hydrogen sulphide in the sinkhole is directly related with the changes in  $[N-NH_3]$  ( $F_{s_{1,10}} = 10.56$ ,  $P = 0.009$ ), an association previously unreported for an anchialine system.

The greatest  $[N-NO_3]$  recorded was  $24 \mu M$ , similar in magnitude to that found by Pohlman *et al.* (1997, in press), but the concentration was still increasing with depth at the lowest sample. However, data from 1994 (Yager & Humphreys 1996) suggest that it will decline to ca.  $4 \mu M$  at a depth of 14 m (similar levels of  $N-NO_3$  were recorded at 6 m in 1994 and 1997, respectively  $1.07$  and  $1.43 \mu M$ ). The  $[N-NH_3]$  is inversely related to the  $[N-NO_3]$  ( $F_{s_{1,10}} = 14.82$ ,  $P = 0.003$ ). These data are consistent with nitrification (Ward 1986), a chemoautotrophic process when ammonium ( $NH_4^+$ ) is successively oxidised to nitrite ( $NO_2^-$ ) and nitrate ( $NO_3^-$ ), an exclusively aerobic process that takes place in the oxic-anoxic boundary layer (Fenchel & Finlay 1995:219). Similar data have been recorded in an anchialine system in Quintana Roo, Mexico (Pohlman *et al.* 1997, in press:11).

An inverse linear relationship between nitrate concentration and salinity was found in an anchialine system by Iliffe *et al.* (1984) and this generally implies conservative mixing and a single freshwater source (Burton & Liss 1975). The opposite relationship between  $N-NO_3$  and salinity was recorded in Bundera Sinkhole ( $F_{s_{1,10}} = 112.1$ ,  $P < 0.001$ ). As the concentration of  $N-NO_3$  in seawater is less than in the sinkhole (data in Yager & Humphreys 1996: Table 1) then the increased concentration of  $N-NO_3$  from 6 m to 14 m depth suggests that  $NO_3^-$  is accumulating in the anchialine system. This finding implies that nitrogen fixation is occurring in the anchialine system and is consistent with the data in Fig 3A) and with the findings of Pohlman *et al.* (1997, in press:11).

#### Microbiotic environment

Much of the surface of Bundera Sinkhole was covered by algal mats at low tide, but at high water these became submerged and the water appeared clear. So, light

penetration into the cave will vary both diurnally and with a superimposed lunar periodicity. In the water column immediately above the thermocline are globular veils characteristic of *Thiovulum* bacteria, but not identified, and which typically enclose regions of elevated sulphide concentration (Fenchel & Finlay 1995). On the sediments are motile filaments (characteristic of *Beggiatoa* spp), which occur mainly in sediments, and therewere attached filaments which occur as 'aufwuchs' on solid surfaces (commonly *Thiothrix* spp).

White filamentous bacteria were photographed moving out of sediments, collected from around the thermocline in Bundera Sinkhole, up the walls of the collecting vessels, presumably tracking the vertical position of the oxygen-sulphide boundary (Fenchel & Finlay 1995:188). Observations on the bacteria samples from Bundera Sinkhole (PD Franzmann, Centre for Ground-water Studies, Wembley; pers. comm.) showed that in samples of globular bodies in the near surface waters there were some short rods, and a number of dinoflagellates (*Gymnodinium*-like) but no filamentous bacteria. At a depth of 8.3 m (at the upper oxygen minimum and where the redox value again just becomes positive) wall samples contained many unsheathed bacteria. There were also many filamentous bacteria (*Sphaerotilus*-like: Clamydobacteriales; *Beggiatoa*-like: Beggiatoaceae and some sulphur crystals) including many large segmented filaments (*Thioploca chileae*-like: Beggiatoaceae), and some eukaryote cysts and diatoms. At a depth of 10.4 m (near the second oxygen maximum) there were limited numbers of rod bacteria, large segmented filaments (*Thioploca chileae*-like  $>10 \mu m$  in diameter), and some larger eukaryote cysts. At a depth of 30.1 m (at the lower oxygen maximum) there were many sheathed bacteria, *Sphaerotilus*-like (some with Fe-III on sheaths), and many rods and some spore-formers. *Sphaerotilus* spp are able to deposit iron on the sheath but sulphur is not deposited in the cells. However, they are considered to be chemo-organotrophic, not lithotrophic, organisms utilizing readily biodegradable substrates.

*Beggiatoa*, the sister taxon to *Thioploca* (Teske *et al.* 1995), and *Thiothrix* (which was not identified in these samples) are large white gliding filamentous sulphur oxidizing bacteria (chemolithoautotrophs) that may form mats in some anchialine systems as well as at deep sea hydrothermal vents and cold seeps. *Beggiatoa* are profuse in areas that are high in sulphide and combined nitrogen but low in dissolved oxygen (Ahmad *et al.* 1999) and their presence can be an indication of advanced eutrophication (Rosenberg & Diaz 1993). They are mixotrophic and some strains harvest sulphide oxidizing it to sulphate using oxygen as the electron acceptor (Hagen & Nelson 1997) but, as high concentrations of nitrate may occur in the vacuole (McHatton *et al.* 1996), it has been suggested that the oxidation of hydrogen sulphide, or endogenous stores of elemental sulphur, are used for the respiratory conversion of nitrate to ammonia.

*Thioploca* (and the recently described giant bacterium *Thiomargarita namibiensis*; Schulz *et al.* 1999) are common on continental shelves (Huettel *et al.* 1996) and belong in a small class of bacteria known to link the sulphur and nitrogen cycles. *Thioploca* spp and *Beggiatoa* spp inhabit aquatic environments at the oxygen-sulphide transition and where the negative chemotactic reactions to oxygen

and sulphide are thought to keep both *Beggiatoa* and *Thioploca* at the optimal position for growth (Huetzel *et al.* 1996).

There is a similarity between the upper 8 m of the profile in Bundera Sinkhole and that found at a microscale (4 cm) in marine sediments. In the sediments nitrate is continually replenished in the oxic zone by nitrifying organisms oxidizing the outward (up and down) diffusion of ammonia from the centre of the anoxic ( $H_2S$ ) zone. In the dark, sulphide, thiosulphate and elemental sulphur are oxidized primarily by chemoautotrophic sulphur bacteria, most of which store sulphur and they therefore appear white in reflected light, hence the term 'white sulphur' bacteria. Most inhabitants of the microbial mat are motile and the chemolithotrophic sulphide oxidisers, such as *Beggiatoa* and *Thiovulum*, track the vertical position of the oxygen-sulphide boundary during changes in light intensity and they may even leave the sediment to form a white haze in the water, showing that the anoxic zone has risen above the sediment surface (Fenchel & Finlay 1995:186). If the anoxic zone reaches the photic zone then a characteristic purple sulphur bacteria is found immediately below the oxycline grading to a black sulphidic sediment. This general sequence of white motile colonies, through purple to black occurs associated with the oxycline in Bundera Sinkhole. In addition to areas of white sulphur bacteria in Bundera Sinkhole, were purple bacteria as typically found immediately below the oxycline if the anoxic zone reaches the photic zone (Fenchel & Finlay 1995). Below these areas were black sulphidic sediments on which the blind gudgeon *Milyeringa* were observed.

Concentrations of bacteria have also been reported from Cenote Angelita in Mexico, an anchialine sinkhole that, like Bundera Sinkhole, lacks significant water circulation. Degradation of organic matter, falling from the forest above, led to the production of  $H_2S$  that was utilized by the bacteria floating above the layer of 250 mM  $H_2S$  at the halocline (Pohlman *et al.* in press).

#### Anaerobiosis and biotic effects

Sulphide is very soluble in water and its dissociation characteristics are such that, at the pH values typical for natural waters, most of it is in the form  $HS^-$ . Since the gaseous form enters cells more easily than the ionized forms, the toxicity increases with decreasing pH. As it binds to cytochrome c oxidase and acts as a respiratory inhibitor, sulphide is extremely toxic to aerobic organisms and exposure to even micromolar concentrations is lethal for species lacking detoxification mechanisms (Fenchel & Finlay 1995:170).

Crustaceans are generally sensitive to anoxia and to sulphide and in sediments all crustaceans are confined to the oxic layers (Fenchel & Finlay 1995:167). Nonetheless, sulphide ecosystems (sulphureta) often contain populations of high density and surprising diversity in the vicinity of aerobic environments where nitrate and sulphate are regenerated from ammonia and sulphide through oxidation with  $O_2$  (Fenchel & Finlay 1995:217). Epigeal crustaceans may be extremely sensitive to both hypoxia and to the toxic effects of hydrogen sulphide (Hagerman & Vismann 1995). However, the fauna in Bundera Sinkhole apparently does not avoid areas where the DO is below 2.0 mL  $L^{-1}$ , and live below and between layers of high  $[H_2S]$  (Fig 3B). The mechanisms by which

the crustaceans and the fish survive these apparently inhospitable waters remains to be investigated.

Owing to the importance of low oxygen level in palaeoecology (Fischer & Arthur 1977; Kaiho 1991) and the biology of the water/substrate interface and of sediments (Fenchel & Riedl 1970), a terminology has been developed to describe anaerobic conditions which is dependent on both the discipline and the type of organisms involved (Tyson & Pearson 1991; Fenchel & Finlay 1995). The division criteria are ecological and physiological, chemical and practical, respectively the avoidance of animals of concentrations below 2.0 mL  $L^{-1}$  DO, nitrate reduction below 0.2 mL  $L^{-1}$  DO, and the limit of the accuracy of the Winkler method of 0.2 mL  $L^{-1}$  DO (Sket 1996).

Sket (1996) was uncertain whether the deoxygenated layers in anchialine caves were completely devoid of oxygen, or only very hypoxic, because of the problem of measuring oxygen precisely under these difficult conditions, a problem exacerbated by the presence of hydrogen sulphide. The water column in Bundera Sinkhole probably covers the full range of oxic conditions; supersaturated by photosynthesis near the surface, through dysoxic (2.0-0.2 mL  $L^{-1}$ ), suboxic (0.2-0.0 mL  $L^{-1}$ ) to anoxic (0.0 mL  $L^{-1}$ ) conditions associated with the hydrogen sulphide layers.

It would appear that the anaerobiosis in Bundera Sinkhole is caused by mechanisms typical of those found to occur in the upper few centimetres of sediments or to other illuminated aquatic microbiotic communities that contain steep gradients of oxygen and sulphide, such as those associated with black band disease in corals (Richardson 1998). I hypothesise that in the upper photic part of the sinkhole, photosynthesis by the algae and cyanobacteria oxidizes the environment during the day and the organic material they produce is broken down by aerobic heterotrophs, restoring the redox balance. However, light intensity falls with depth and the temperature increases markedly at the thermocline resulting in both a reduction in oxygen production and an increase in reaction rate. Furthermore, this is associated with a local stability as oxygen transport will be reduced in the absence of convection across the thermo-halocline. Together, these result in local depletion of oxygen. As degradation by anaerobic microbes takes over, the metabolic end-products (e.g. sulphide, hydrogen) will render the surroundings chemically reducing. These changes are most likely to occur in places where there is a large import of dead organic material — the photic zone of Bundera Sinkhole is eutrophic producing large amount of organic matter — and protection from turbulent or advective transport of water, respectively by the sinkhole walls and the thermo-halocline. While this process is well supported by the data for the upper sulphide layer, the uniquely repeating and sulphidic (and roughly concordant redox and oxygen minima) layers in Bundera Sinkhole (Fig 2E) is poorly documented and less obvious in origin. Nonetheless, it would seem that the typical remiped crustacean community is only found below the second sulphide layer (Fig 3B), and which itself is not associated with a thermo-halocline (Fig 2A).

#### Stable isotope analysis

The data for the stable isotope enrichment of both carbon and nitrogen are given in Fig 3C. They are

grouped according to  $C_3$  and  $C_4$  plant metabolism (*Acacia* and *Triodia* respectively), algae, shrimps (Atyidae), predators (the blind gudgeon, *Milyeringa veritas*, and gerrids [one pooled sample]). Analysis is shown of pooled samples of tubificid worms and the mollusc *Iravadia* sp that inhabit the algal layer. Finally, the 'line sediment' represents the accumulation of particulate organic matter settling over more than a year onto the guide line at depths of 17.5 m and 21 m.

The overt organic input is determined by the local vegetation which in this arid area is hummock grassland ( $C_4$  plants) with few scrubs and annuals ( $C_3$  plants) at the time of sampling. Hence, the vast majority (>90%) of local vegetation coverage comprises  $C_4$  plants on the shallow sandplain overlying a marine terrace cut into limestone which deeply covers the cave (Fig 1). Given the scant and episodic rainfall in this region the downward percolation of organic matter through the sand and limestone overlying the cave is likely to be sparse. There are a few plants in the sinkhole of both  $C_3$  and  $C_4$  plants but the predominant vegetation is the thick algal community in the water of the sinkhole and which sometimes covers the surface and extends to several metres below the water level. It mainly comprises the algae *Rhizoclonium tortuosum* (Dillw) Kuetz (Chlorophyta: Cladophoraceae) and *Lamprothamnium papulosum* (Wallr) J Gr (Charophyta: Characeae) and is thickly populated by the mollusc *Iravadia* sp. Large colonies of bacteria occur near the thermo-halocline both in the water column and on the walls and floor of the cave.

**Carbon.** The  $^{13}C$  values for the  $C_3$  and  $C_4$  plants are within normal limits which have median values respectively of -27 ‰ and -11 ‰ (Lajtha & Marshall 1994). While the values of  $^{13}C$  for algae in Bundera Sinkhole are heavy compared with most values in the literature, they are not outside recorded limits. Values of  $^{13}C$  in marine algae are between -19 to -24 ‰ and are affected by the uptake of dissolved inorganic carbon in planktonic photosynthesis (Peterson & Fry 1987). In fresh water values of  $^{13}C$  vary widely depending on the source of dissolved  $CO_2$ ; where respiration inputs are strong, the  $\delta^{13}C$  values for dissolved inorganic carbon may approach -20 ‰, with algae reaching -45 ‰ owing to fractionation during carbon uptake (Peterson & Fry 1987). In algal cultures  $\delta^{13}C$  variations ranging from -29.7 ‰ to -5.5 ‰ have been reported (Falkowski 1991), with about one third of this variation attributed to changing concentrations of  $CO_2$  but the very high values near -5 ‰ were attributed to the active uptake of inorganic carbon (Goericke *et al.* 1994), hence these values could be influenced by the carbonate chemistry of the karst system itself. Anchialine systems, such as Bundera Sinkhole, have attributes of both freshwater and marine systems (owing to the marked physico-chemical stratification of the system, these occur in the same location) but the relative influence of the marine system using, for example, the  $\delta^{34}S$  distribution (Peterson & Fry 1987), has not been investigated.

**Nitrogen.** The values of  $\delta^{15}N$  for the  $C_3$  and  $C_4$  plants are within normal bounds (Peterson & Fry 1987) but the  $\delta^{15}N$  values for the algae are high, for unknown reasons. While the fractionation of nitrogen isotopes is rarely greater than 2 ‰ in natural systems, it is often large (10-20 ‰ for nitrate or ammonium uptake by aquatic algae and

microbes (Macko & Ostrom 1994). Where nitrate is abundant, as in the surface water of Bundera Sinkhole (Yager & Humphreys 1996), fractionation during assimilation by phytoplankton may lead to a  $\delta^{15}N$  sufficiently heavy (Peterson & Fry 1987) to reach the level found in Bundera Sinkhole. Study of the isotopic fractionation in the cascade of DIN species in the upper water profile of the cave would help resolve these uncertainties.

**SIA and trajectories.** The line sediment, the tubificid worms and molluscs lie within the envelope of an hypothesised source of carbon and nitrogen derived from a mix of  $C_3$  and  $C_4$  plant material and algae (see Figs 3C, 3D). The predators form two groups depending whether they originate from superficial parts of the cave or the deep zone, with the former being 'heavier'; the gerrid data are consistent with this pattern. The atyid shrimps lie outside the hypothesised food mix envelope, being lighter. The trophic trajectories (Fig 3D) suggest that the two predator populations are derived from  $C_3$  plants (two trophic steps removed), or atyid shrimps (one trophic step removed); *Milyeringa veritas* is known to eat the shrimps (Humphreys & Feinberg 1995).

As shown above, there is evidence from the stratification of the DIN species that nitrification was occurring in Bundera Sinkhole. Furthermore, visual observations in the main water body, observation of the characteristic motile behaviour of sulphur bacteria observed *in vitro*, and the presence of  $H_2S$ , suggest that sulphur bacteria are widely present at and below the thermo-halocline and these are likely to be involved in chemoautolithotrophy. Such chemosynthetic biomass is characteristically lighter (more negative  $\delta^{13}C$ ) than photosynthetically derived carbon (Conway *et al.* 1994). While no isotope determinations were made on bacteria, these observations could be verified by stable isotope determinations of the bacteria.

One explanation for the lightness of the atyid shrimp data is that they are utilizing chemoautotrophic bacteria for part of their food source: atyid shrimp have a brush-shaped tuft of setae in the first two pereopods with which they brush the substrate while feeding (Bailey-Brock & Brock 1993). Evidence in support of chemoautolithotrophy in anchialine ecosystems is presented by Pohlman *et al.* (1997 in press) for an anchialine cave in Mexico.

#### Comparison with other areas

The high surface salinity and the complexity of the profile in Bundera Sinkhole may be associated with the aridity of the area and hence minimal surface input and low ground-water flow of ca. 0.003 mm s<sup>-1</sup> on the eastern side of the same peninsula. This results in a broad zone of diffusion, 20-30 m thick, between the overlying freshwater lens and the underlying seawater (Exmouth water supply borefield; Consultative Environmental Review; Water Corporation, unpublished, 1996). Bundera Sinkhole probably lies at the extreme seaward side of the system where only the diffusion zone is represented and thus exhibits only the brackish/seawater interface seen in the sinkhole.

Most anchialine systems that have been examined have a freshwater lens overlying sea water (Iliffe in



press); the freshwater lens may be thick and increase in depth inland, for example, on the Yucatan Peninsula 2 km inland it is 10 m thick at sinkhole Abejas and 10 km inland it is 20 m thick at sinkhole Vaca Ha (Iliffe in press). At Wonderland Cave, Bermuda, brackish water overlies seawater, and this cave is most comparable to Bundera Sinkhole in its salinity profile (Iliffe in press: Fig 3).

A summary of published physico-chemical profiles of anchialine systems is given by Humphreys *et al.* (1999); Bundera Sinkhole is the deepest, and probably the smallest, of these. In Bundera Sinkhole there is a polymodal DO depth profile, a form shown in none of the published DO depth profile, all of which exhibit a unimodal DO depth profile. By inference it suggests that the other systems probably have a single H<sub>2</sub>S layer should one be present.

The DIN species data for Bundera Sinkhole (Fig 3A) are very similar to those reported by Pohlman *et al.* (in press) in a cave where they obtained strong evidence, using stable isotope analysis, that chemoautotrophic energy fixation was occurring. This means that such systems, rather than being totally dependent on imported (allochthonous) energy sources, as is the general model for cave systems, they can derive part of their energy *in situ* from autochthonous energy fixation. This has considerable implications to anchialine systems because it means that energy may be available broadly through anchialine systems, even where the ground-water is covered by impermeable ceilings. In this context, it is worth noting that sulphide-oxidising mixotrophic *Thiothrix* and *Beggiatoa* bacteria, which may be primary producers using sulphide-based chemosynthesis (Brigmon *et al.* 1994; *cf.* Kane *et al.* 1994), occur widely in anchialine and other ground-waters (Yager 1991a; Brigmon *et al.* 1994), as well on deep-sea hydrothermal vents (Belkin & Jannasch 1989).

The physico-chemical environment in Bundera Sinkhole is very complex and this complexity is associated with biogeochemical processes that are likely to be of fundamental importance to the maintenance of the unique community contained in this anchialine cave. SCUBA diving alters the vertical stratification of the physico-chemical environment in the sinkhole (Humphreys *et al.* 1999) and the effects of this may be disruptive to the unique anchialine community inhabiting the cave. As some of the fauna associated with such anchialine systems have only ever been collected by divers, remipedes for example, then diver access is essential for biological work. However, this should be limited and where possible be conducted using rebreathing equipment. There is a lack of basic data on the gross physico-chemical environment in Bundera Sinkhole, on ground-water movement, temporal changes in the profile resulting from the effects of episodic rainfall on surface input as well as ground-water flow. A diver-free profiling system needs to be established to determine long term changes, particularly the establishment and maintenance of the complex redox profile, its associated chemolithotrophic organisms and the significance of these processes to the remipede community.

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