



Research article

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Colonization of a new habitat: The case of the Dead Sea sinkholes – preliminary observations

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A B S T R A C T

The recent process of sinkhole formation in the Dead Sea area provides a unique opportunity to observe the populating of new, pristine niches in extreme environments. Four sinkholes in the Ein Gedi vicinity were chosen for a preliminary ecological study. Sampling was carried out over a 14-month period in 2009-2011. Light microscopy was employed for observations; taxonomic guides and scientific literature were used in an attempt to identify the species at the level of genera. Each sinkhole was found to have its own ecological signature and history, characterized by different succession patterns and affected by its own dynamic physical/chemical milieu. In three out of four sinkholes, microbial/algal mats served as the matrix for increasing biological diversity. In a highly saline sinkhole (SH4), a succession pattern was observed where Bacterial/Archaeal dominance was succeeded by a mono-species Cyanobacteria mat (*Phormidium sp.*). In contrast, in SH2, mats were rudimentary and dominance shifted from a unicellular Chlorophyte (*Dunaliella sp.*) in winter to a unicellular Cyanobacteria (*Aphanothece sp.*) in summer. Three types of mats developed in the sinkholes: (1) filamentous Cyanobacteria-based; (2) Baccillariophyta-based, and (3) mixed. Salinity seemed to play a crucial role on species diversity and composition, but minor seasonal fluctuation did not seem to have a major effect. Some species might be unique to the sinkholes, like the miniature crab and the diatom/microbial associations. Most other organisms, like *Aphanothece* and *Dunaliella*, inhabit other haline water sources in the area that could have provided the genetic pool for colonizing the sinkholes. Further study of the sinkhole environment would help elucidate the patterns of ecological succession and dynamics in new hyper-saline water bodies.

Keywords:

Sinkholes
Dead Sea
Colonization
Diatom mat
Cyanobacteria mat
Halophiles

Abbreviations: SH = sinkhole

1. Introduction

The recent process of sinkhole formation in the Dead Sea area has provided us with the opportunity to observe the populating of new, pristine niches. This opportunity, to witness self-organization of aquatic ecological communities in one of the most extreme environments in the world, is priceless and should not be missed. Sinkholes are holes in the ground formed by either a gradual natural process of erosion or, conversely, through a sudden collapse of a cavity's roof. They "provide a home for unusual plant and animal communities and a special link between the earth's surface and our underground resources" (Friend,

2002). Most sinkholes are formed in karst and many are flooded (Palozzi et al., 2010; Sahl et al., 2010). Some sinkholes are actually submerged in lakes and oceans while harboring distinct micro ecosystems; others are entirely terrestrial and sometimes serve as faunal traps for animals (Pokines et al., 2011). Even terrestrial sinkholes usually have at least a moist bottom and are connected to underground water in various ways (Friend, 2002). Sinkholes can be very deep; the deepest sinkhole in the Dead Sea area reaches about 20 m (Hebrew University Wiki, 2011).

The formation of the sinkholes in the Dead Sea area is generally ascribed to the decline of the lake level and the

resulting regression of the coastline, ensuing in a parallel drop in underground water level (Arkin and Gilat, 2000). As a consequence, the continuous salt layer embedded in the sedimentary rocks along the Dead Sea coast is penetrated from underneath by pressurized under-saturated water flowing in the descending aquifer; the dissolution of the salt results in underground cavity formation (Abelson et al., 2003, 2006; Frumkin and Raz, 2001). A wet clay layer flows into the forming cavity, leaving the dry clay layer hanging overhead. The plummeting sea level creates or reactivates existing faults and fractures. The generated stresses destabilize the overlying layers of finely grained silt and clay, culminating in the formation of open sinkholes (Shoval, 2003). Merging of sinkholes, as seen in two of the sinkholes monitored in our study, may follow as the process of salt dissolution continues (Shoval, 2003). It can also follow from the action of Syrian-African Rift faults (Abelson, 2003). Since 1980 the number of sinkholes has increased in the Dead Sea area, mostly in flat areas of the south coast of the Israeli side.

Dead Sea sinkholes are usually flooded, though some have dried up or fill up periodically. They often contain brine that was left over by the receding Dead Sea and was trapped within the surrounding sediments (Katz and Starinsky, 2009). Once in the sinkhole, the brine evolves chemically through evaporation. Sinkhole brines were found to be compatible with the evaporative evolution of Dead Sea water and can display salinities of up to ~500 g/l (Ibid). It is also likely, based on how the sinkholes were formed, that some of the water in the sinkholes could have originated from the mountain aquifer. It is yet to be investigated if such underground connections might account for the introduction of some of the species in the sinkholes.

Since the Dead Sea sinkholes are of the collapse type (Friend, 2002) and are formed abruptly, they are good candidates for studies of colonization and succession. Creation of “islands” in such a short time allows us to study “island biogeography” in a nutshell. Sinkholes were found to be important in keeping genetic diversity (Rivera and Aide, 1998) as well as breeding new species (Koblmüller et al., 2008). They serve as stepping-stones for species distribution (Cervantez-Martínez et al., 2005), and as propagule traps (Rivera and Aide, 1998). As a new phenomenon in an established ecosystem, they are sites of ecological succession in a small scale and may serve as a window to the unseen biological diversity of the wider region.

Not ruling out the possibility of an aquiferian source for some sinkhole organisms, other likely sources of species introduction into the sinkholes are the Dead Sea itself, its sediments, coastal pore populations and the various water bodies in its drainage

basin. Until 1979, the Dead Sea was a meromictic hypersaline lake with anoxic sulfuric deep waters and a seasonally varying mixolimnion. Due to the decrease of water influx into the lake since the beginning of the 20th century, the water budget became negative resulting in increasing surface water density and leading to an overturn in 1979. Since then, the Dead Sea remained a holomictic lake, except following exceptionally rainy seasons (Ionescu et al., 2010). Due to NaCl oversaturation, halite is deposited and divalent cations such as Mg⁺⁺ dominate. Total dissolved solids (TDS) reach about 347 g/l. The unique mineral composition is dominated by MgCl₂ (50.8%), CaCl₂ (4.4%) and KCl (4.4%). NaCl is only about 30.4% of the total TDS compared with about 97% in the world's oceans (Steinhorn, 1983).

Life in the Dead Sea was traditionally considered to be limited to Archaea, Bacteria, Cyanobacteria and Chlorophyta of the genus *Dunaliella* (Oren, 1999; Bardell, 2010). Most of those species are endemic to this environment, characterized by high salinity and very high positively charged ion concentrations. The food chain in the Dead Sea is considered one of the simplest (Bardell, 2009), with *Dunaliella* as the primary producer, and Archaea and Bacteria dependent on it for organic nutrients. Twenty two species of fungi act as degraders (Oren, 1999). Recently, rich and diverse microbial communities were found in and around springs discovered at the bottom of the sea in several locations (Ionescu et al., 2012). These communities consist of phototrophic bacteria, including Cyanobacteria, sulfide oxidizers, sulfate reducers, nitrifying bacteria, and iron reducers and oxidizers. The source of the microorganisms in the springs and their vicinity was determined to be the Dead Sea sediments themselves and the shoreline pore water, and shown not to originate in the aquifer (Ibid).

Additionally, organisms could have reached the sinkholes from the rich communities found in the larger drainage area using various dispersal methods (Kristiansen, 1996). Thorough reviews and lists of organisms found in the Dead Sea basin are given by Oren et al. (2008) for Cyanobacteria and microalgae, and by Ehrlich (1995) for Bacillariophyta. Chlorophyta were not studied methodically (Oren, 2008), but genera commonly found include *Scenedesmus*, *Pediastrum*, *Ulothrix*, *Dunaliella* and *Chlamidomonas*.

Much of the previous studies carried out on the western bank of the Dead Sea were done on artificial salty water bodies such as the evaporative ponds of the Eilat salt plant (Sorensen et al., 2005; Rehakova et al., 2009), the Ein Boqeq and Beit Ha'arava solar ponds (Dor and Ehrlich, 1987; Dor and Hornoff, 1985a,b) and the Ein Tamar experimental ponds near the Dead Sea

(Barinova et al., 2004). Some of this work describes the early stages of colonization and succession in such ponds: Dor et al. (1992) followed colonization of a brand new artificial solar pond and found that the pioneer species appearing only several weeks after construction were unicellular Cyanobacteria represented by *Aphanothece halopytica*, and several diatoms, mostly *Navicula duerrenbergiana*, *Nizchia rostellata* and *Amphora coffeaeformis*. After a year of growth, the stromatolite mat was about 5-6 mm thick. At this stage, anoxygenic filamentous *Phormidium hypolimneticum* appeared underneath the mat and the odor of H_2S was noticed. The fact that no higher trophic levels were found in those ponds was attributed to the unique composition of Dead Sea brine, inhibiting the development of Metazoan browsers. In a study of 3-5 year-old solar ponds, Dor and Ehrlich (1987) listed 25 taxa of diatoms and Cyanobacteria and one species of Chlorophyta (*Euglena*). Since no multi-layer mats were formed even 5 years after construction, the population was considered by the authors to still be in a pioneering stage.

In view of the described research of colonization of superhaline bodies around the Dead Sea, the sinkholes are of particular interest, as these ecological systems were formed naturally and their ecology is affected almost entirely by natural processes, such as colonization and dispersal. Also, the majority

of the artificial ponds studied are very shallow, and some, like the Ein Tamar ponds are very small, while the sinkholes are generally deeper and have different dimensions. The Ein Gedi Field School area was chosen because of its abundance of sinkholes, its interest to the public and its accessibility.

The following preliminary observations portray an interesting picture of population dynamics in four selected sinkholes in the Ein Gedi Field School area over a 14-month period in 2009-2011. The goal was to see the sinkhole communities in the context of the wider Dead Sea region and to follow them over the experimental period in terms of species diversity and proliferation as well as over the seasons. The hypothesis was that biodiversity and trophic levels would increase with sinkhole age and that this diversity would be affected by the seasons as well as by non-biotic factors particular to each sinkhole.

2. Materials and Methods

2.1. Sample collection

The sinkholes chosen are located in the Dead Sea area (Fig. 1a) near the Ein Gedi Field School in close proximity to the sea (Fig. 1b). Pictures of sinkhole 1 and sinkhole 2 are shown in Fig. 2.

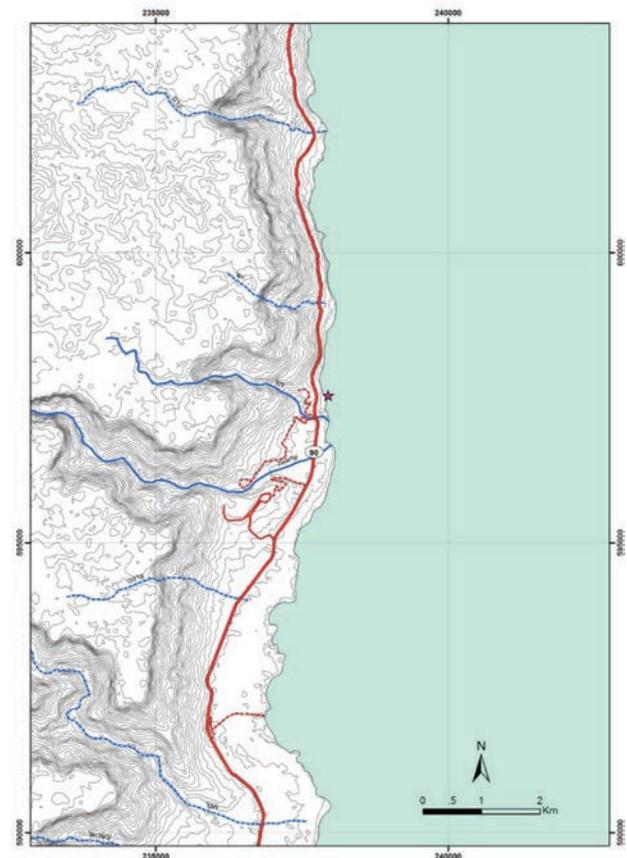


Figure 1: Sinkhole location: (a) in the general Dead Sea area (b) in the specific shore segment (*starred)



Figure 2: Left: Sinkhole 1, Right: Sinkhole 2

Sampling was done sporadically over a 14-month period (Table 1). Due to contamination at SH2, sampling was discontinued after 25/08/2010. It was noted that by 16/01/2011, sinkholes 2 and 3 which were initially separate have merged and were thereafter sampled as a single site.

Table 1: Sampling dates by sinkhole

	SH 1	SH 2	SH 3	SH 4
23-Nov-09	+	+	-	-
15-Jan-10	+	+	-	-
9-May-10	+	+	+	+
23-May-10	+	+	+	+
11-Jun-10	+	+	+	+
25-Aug-10	+	+	+	+
3-Oct-10	+	-	+	+
17-Oct-10	+	-	+	+
16-Jan-11	+	+		+

Samples were taken from surface water in plastic bottles and brought to the Arava and Dead Sea Science Center lab in Maale Shoharut School, Yotvata. Acidity (pH) and electrical conductivity (EC) were measured in the Yotvata lab. Dilutions with deionized water were executed to determine the EC (which is not identical to total dissolved solids [TDS] – weighing the residuals found in water, a procedure that was not undertaken). These measurements were taken at room temperature (Fig. 3).

2.2. Identification of organisms

A Motic BA310 microscope was used for sample analysis; photographs and videos were taken using the attached Moticam 2500 camera. Additionally, some samples were enriched with modified F/2 medium (0.02M KNO₃, 0.0015M KH₂PO₄, 0.00166M MgSO₄, 0.00065 M SiO₂, 0.012 g/l Fe-EDDHA, 0.0006 g/l Mn-EDTA, 0.0003 g/l Z-EDTA, 0.00005 g/l Cu-EDTA, 0.00343M Mo) in Red Sea water ("Red Sea Fish Farm", Israel) at various salinities, and bubbled, initially with air, and then with 0.5% v/v CO₂ (~2 l/minute).

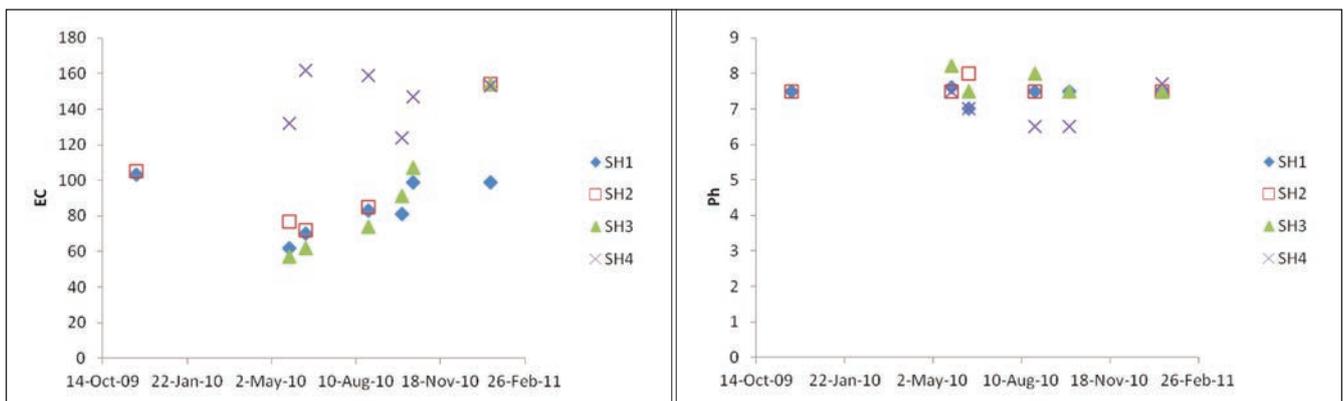


Figure 3: Abiotic parameters at the various sinkholes through the sampling period: (a) Electrical conductivity (EC), (b) pH

Taxonomic guides and scientific articles (e.g. Chrétiennot-Dinet, 1990; Dor and Ehrlich, 1987; Dor and Hornof, 1985a; Kilroy, 2004; Nubel et al., 2000; Oren et al., 1995; Protist information server, 2013; Round et al., 1990; Sorensen et al., 2005) were used to help with classification to the level of the Recognizable Taxonomic Units (RTU), which ranged from genera to phyla (Ward and Stanley, 2004). The term "bacteria" will be used in this article for both Bacteria and Archaea for lack of tools to distinguish between the two domains.

2.3. Data analysis

Since this is a preliminary study that is only addressing the issue of the relative importance of different groups in the newly established sinkholes, our aim was not to quantify the abundance of the organisms, but to relate to the dominance of different groups. Attempts to answer how to accurately quantify cells from a particular microbial group have produced highly conflicting results (Lloyd et al., 2013). Similarly microscopy-based methods used for determining the abundance of Protista often produce a variety of resolutions (Weber and Pawlowski, 2013). So while we were not able to determine the exact number of individuals, we estimated their relative quantity, or performance. Relative species quantity relates to how common a species is relative to other species in a sample or a given location and it relates to the evenness of the community (Hubbell, 2001; McGill et al., 2007). Ecologists use data of species relative abundance to infer information about the mode of interaction and type of relationship among different species in a given community (Ferrier and Guisan, 2006) and it can be used to estimate ecological disturbances and other ecological processes (Possingham et al., 2001, Nidal et al., 2009).

The relative quantity of each RTU was assessed on a level of 0-3 (3 indicating high abundance) for each collection date and each sinkhole, according to microscopic inspections. We based our estimations on the ACFOR (Abundant, Common, Frequent, Occasional, Rare) system (as in Brendan et al., 2006) indicating order of magnitude of densities, in which: 0 indicates not present, 1 to very few individuals, 2 to occasional or frequent and 3 to common or abundant.

Average quantities of the totals and of each RTU in each sinkhole at each date were tallied. No statistically significant tests were carried out since quantification was only carried out in relative values. The analysis was done once on the RTU level, which is the lowest taxonomic level of identification possible and separately on the phylum level in order to examine which are the dominant phyla in each sinkhole. To check

the similarity of the sinkholes regarding their composition, we compared the RTU (mostly genera) using a Bray-Curtis dissimilarity index, that was converted to a similarity index (1-BC).

3. Results and observations

3.1. The physical factors

3.1.1. Temperature – The sinkholes do not appear to be connected to underground thermal water sources: therefore water temperatures are in balance with air temperatures. The highest temperatures recorded were 37 - 39 °C at SH1 on August 2010.

3.1.2. Salinity – Electrical conductivity (EC) was used as a measure for salinity (see comment above). The EC varied widely over time and from one sinkhole to another (Fig. 3a). The values are for diluted samples and should therefore, be considered in relative terms. For comparison, EC of the Dead Sea water collected near the hotel area in Ein Boqeq on June 2nd, 2010, was measured to be 189 mS·cm⁻¹ following a 10-fold dilution.

Electrical conductivity in SH1 and SH2 decreased towards spring 2010 and then increased towards October 2010, where values as high as 99 and 107 mS·cm⁻¹ were reached in SH1 and SH3 respectively (Fig. 3a). EC continued to rise in SH3, but stayed at this level in SH1. Values of EC in SH1 in October 2009 and October 2010 were similar, indicating a seasonal pattern. Measurements were not taken from SH2 after August 2010 due to an animal corpse found in the water. Salinity at SH4 was very high throughout the sampling period with minor fluctuations.

3.1.3. pH – The pH in sinkholes 1,2 and 3 was neutral to slightly alkaline (Fig.3b). The most alkaline pH was measured in SH3 on May 23rd, 2010 (8.5). At SH4, neutral to slightly alkaline pH was measured until June 2010, but between August and October the pH decreased to 6.5.

3.2. The biological findings

When floral and bacterial taxa were taken into account, the highest Bray-Curtis similarity index was found between SH1 and SH3 (Table 2), and the lowest between SH3 and SH4. Similarity indices were generally low. Overall, SH1 and SH3 were dominated by Baccilariophyta, SH2 by Chlorophyta and SH4 was almost exclusively inhabited by the simple groups of Cyanobacteria and bacteria (including Archaea). SH1 had the highest level of activity of microorganisms and flora (Fig. 4a), as well as of fauna and heterotrophic Protista (Fig. 4b).

Table 2: Similarity of flora composition of each sinkhole expressed as a Bray-Curtis index

Sinkhole	1	2	3	4
1	1	0.26	0.56	0.25
2		1	0.32	0.26
3			1	0.18
4				1

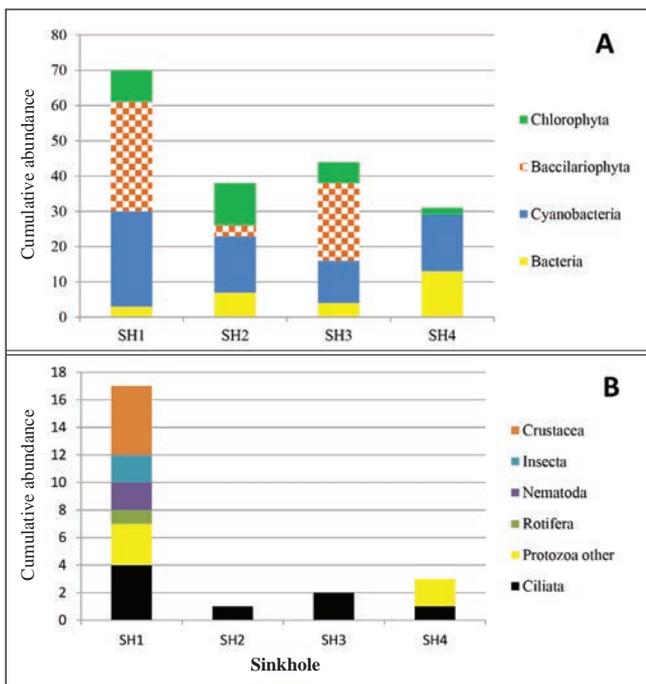


Figure 4: The relative occurrence of the larger taxonomic divisions in the sinkholes based on semi quantitative estimates – (a) flora, bacteria ; (b) fauna, heterotrophic Protists

3.2.1 SH1 – Sinkhole 1 exhibited the richest ecological environment among the sinkholes sampled (Figs. 4, 5). Intricate mats, created by a network of several species of Pennate diatoms and of filamentous Cyanobacteria formed the base for this relatively stable ecosystem (Fig. 6; vids. 1, 6, 9, 11). The mat served as a host for a variety of unicellular Chlorophyta and Cyanobacteria, Protozoa and Ciliata, and even miniature Metazoa, including Rotifera, Nematoda and Crustacea. The mats sampled floated on the water surface. The species diversity increased in the spring and towards the summer, which also coincided with the reduced salinity (Fig. 3a).

Filamentous algae and Cyanobacteria: Among the filamentous Cyanobacteria, *Halospirulina*, reaching up to hundreds of coils, was prominent (Vids.1, 2, 3, 4), as well as long filaments of *Phormidium*, *Anabaena*, *Oscillatoria* and others (tentative identifications, see Vids.3, 5).

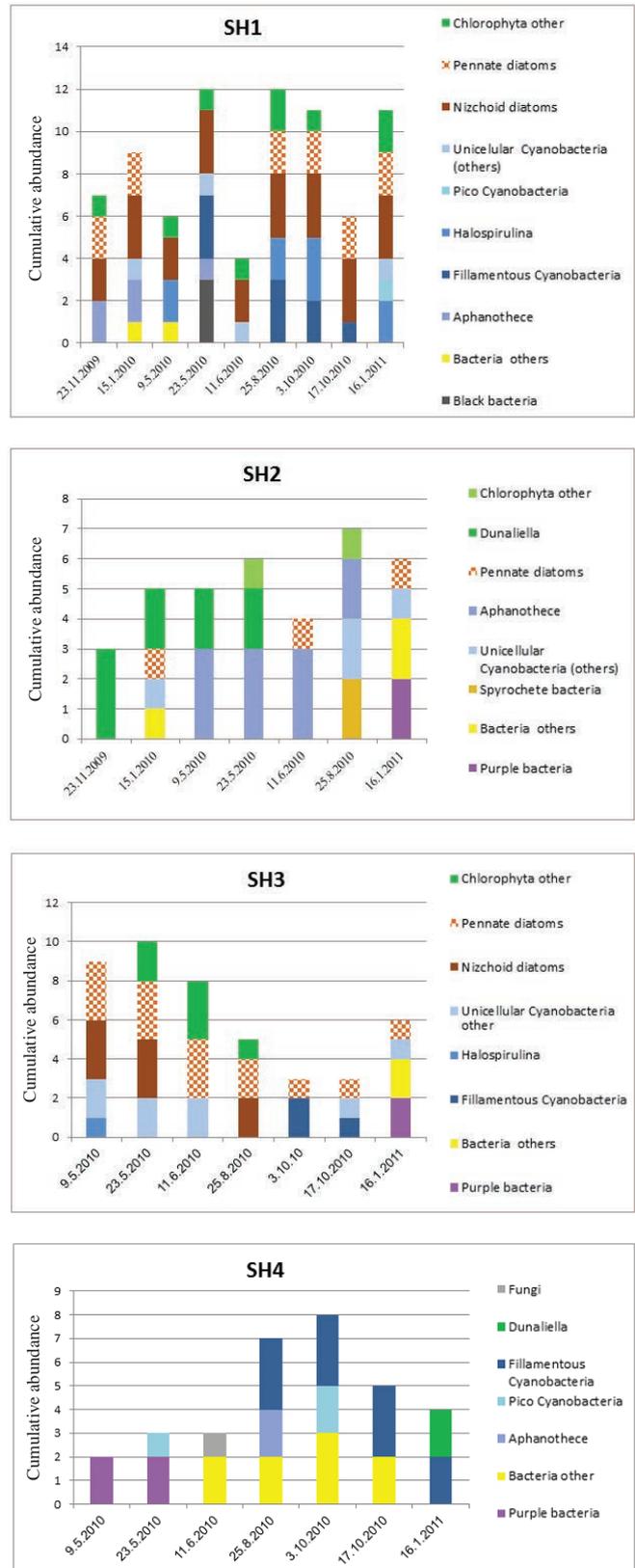


Figure 5: The changes in population composition of the different taxa (RTU, or recognizable taxonomic units) during the sampling period. Bacteria colors were judged by appearance under light microscopy and are not necessarily proper taxonomic identifications. The general term "Bacteria" refers to both Bacteria and Archaea. It should be noted that by January 2011, SH2 and SH3 merged.

Diatoms: Many types of diatoms were observed in the mats and the water column. The vast majority were Pennales, with the majority being Naviculoid or Nitzchioid. Salient in the mats were huge diatoms, reaching up to 400-500 μm with a typical size of about 200 μm (larger readings of up to 1 mm are uncertain), both straight and sigmoid (Fig. 6; Vids. 1, 2, 6, 7 and 8). We tentatively identified the non-symmetrical large diatoms as *Pleurosigma* or *Gyrosigma* spp. It is possible that the very large straight diatoms are an endemic strain or species. A unique and consistent feature of the huge diatoms was the almost ubiquitous association with two types of prokaryotic

cells: long rod-like bacteria that were attached to their outer surfaces, and round green bodies, probably Cyanobacteria, that were seen both inside and outside the cells, and can therefore be considered both epiphytic and endophytic (Fig. 6; Vids.1, 11). Since diatom chloroplasts are yellow-brownish, these greenish bodies must be unicellular organisms in their own right, most probably Cyanobacteria. In addition, several species of smaller Pennate diatoms (about 50-60 μm in length), probably belonging in the *Navicula*, *Amphora* and *Nitzschia* genera, were seen, sometimes in large quantities and in association with the mats.

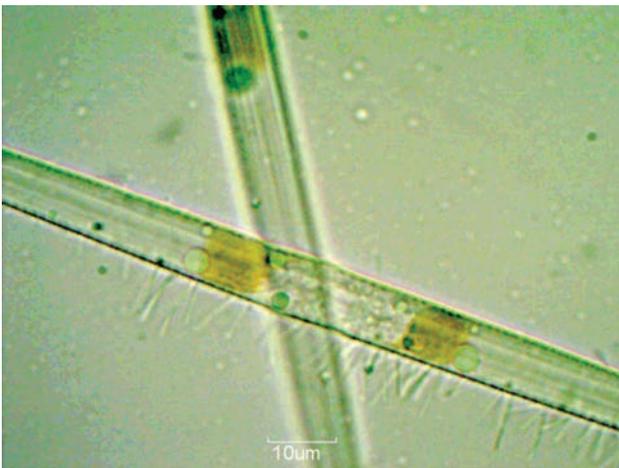


Figure 6: Large Nitzchioid Pennate diatoms with microbial and Cyanobacterial epiphytes and endophytes, straight and sigmoid

Other photosynthetic organisms: Various single-celled Chlorophyta resembling *Chlamidomonas* were observed, as well as various single-celled Cyanophyta like *Synechococcus* and *Aphanothece* (also known as *Euhalothece*) (Fig. 7).

Heterotrophic life: Various Protozoa, including Ciliata were found around the mats, mostly in summer 2010 and

onwards. SH1 was the only sinkhole in which trophic levels higher than Protozoa were observed. Several Metazoan phyla were represented in the mat environment and were abundant in the summer: Nematoda were seen between May and August; mosquito larvae were observed in the spring. Miniature unidentified crabs, reaching 200-400 μm in width, were recorded

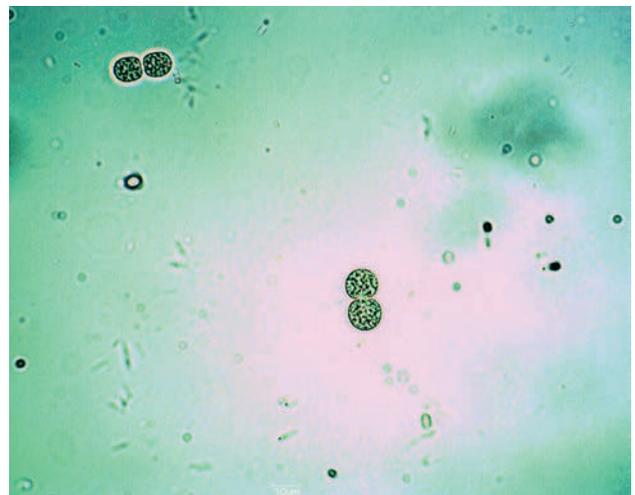


Figure 7: *Aphanothece/Euhalothece* sp. showing the typical transverse division pattern. Granulated cytoplasm could be due to osmo-regulatory substance accumulation

moving and feeding around the mats since August 2010 and all the way through January 2011 (Fig. 8a; Vids. 5, 12, 13, 14). *Artemia salina* also appeared in the mats during the same period (Fig. 8b; Vid.14). Rotifera were sometimes observed (Vids.7, 9).

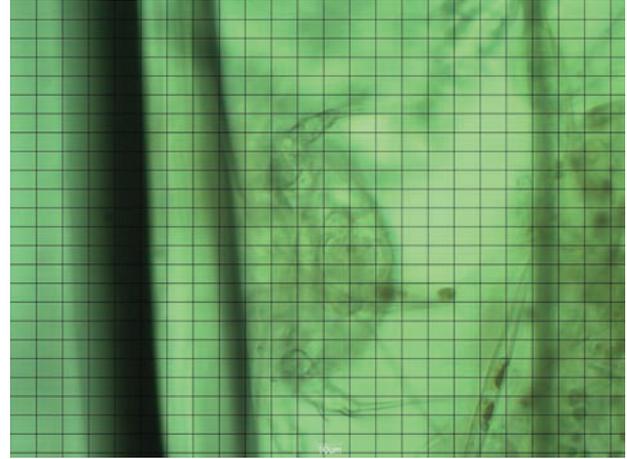
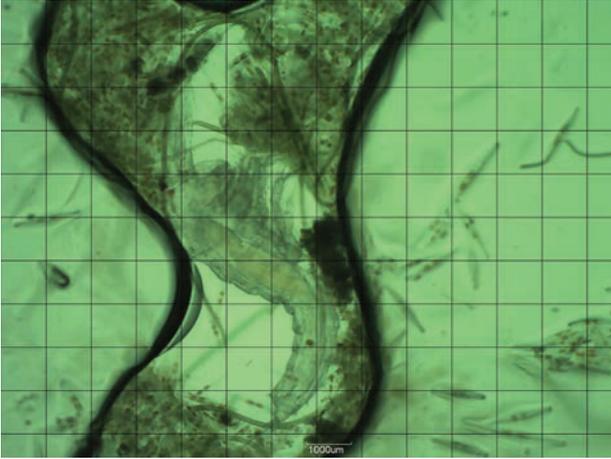


Figure 8: Crustaceans: (a) Microscopic crab in diatomaceous mat. (b) *Artemia salina*. SH1, August 2010

3.2.2. *SH2* – Although pH and salinity patterns in SH2 were generally similar to SH1, SH2 was characterized by a very different micro life to SH1 (Figs. 4, 5). Samples from November 2009 and January 2010 were dominated by *Dunaliella* sp. (Fig. 9). By May 2010, the unicellular Cyanobacteria, *Aphanothece*, became dominant (Fig. 7). The cells, about 7µm in diameter, were almost always seen in transverse division pairs and were highly granulated. No diatoms were seen at that time in SH2 until June 2010, while the neighboring SH1 was diatom-dominated throughout the sampling period. The *Aphanothece* sp. was still prominent in August 2010. *Synechococcus* sp. was observed in addition to other Cyanobacteria in January 2011. Protozoa, and in specific, Ciliata, were also seen in SH2, but no representatives of multi-cellular animal phyla were recorded.

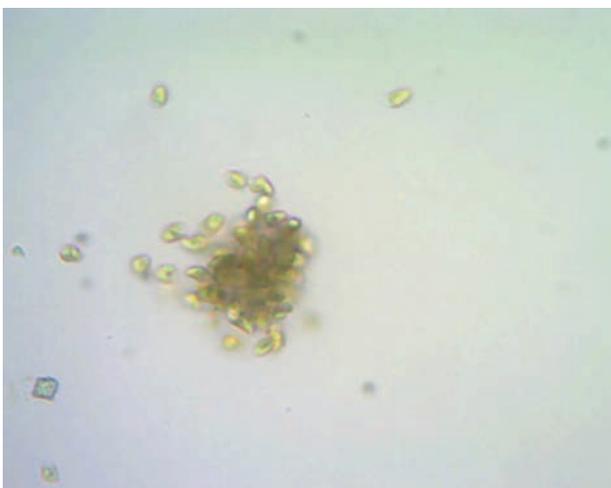


Figure 9: *Dunaliella* sp. in SH2, Nov 25th, 2009

The complexity of a mixed Diatom/Cyanobacteria mat with its associated community can be seen in vid.8. The high mobility of all mat elements can be observed in video 3 and all SH1 videos from the 23.5.2010 and 25.8.2010.

3.2.3. *SH3* – Initially the flora in SH3 was similar to that of SH1 (Fig. 5), but by June 2010 Chlorophytes (*aff. Chlamidomonas*) seemed to have taken over the mat society. By August 25th, 2010, no mats were visibly seen on the water surface, the water was light green and the flora was dominated by *aff. Chlamidomonas*, though huge diatoms were still abundant. In the October 2010 sample, smaller Pennales were more abundant, and by mid-October, when EC values rose to ~107 MS·cm⁻¹ (Fig. 3a), abundant dead organic matter was observed on the water, and small Cyanobacteria seemed to have occupied the niches left by the destroyed mats. The same combination of smaller diatoms and Cyanobacteria persisted following the fusion of SH3 with SH2. Faunal life in SH3 was much poorer than at SH1. No higher Metazoan phyla like Nematoda or Arthropoda were recorded. Heterotrophic life was represented by Protista.

3.2.4. *SH4* – Sinkhole 4 was characterized by a very high salinity and a neutral to slightly acidic environment (Fig. 3a, b). Colonization of this sinkhole was much slower and limited to very few species, the most successful of which were Cyanophyta, either solitary or filamentous (Figs. 7, 9, 10). Initially, the flora consisted exclusively of bacteria, seemingly purple by color, but their identity could not be established with our methods. Purple bacteria oxidize H₂S to elemental sulfur in photosynthesis. They live in anoxic water in lakes and sulfur springs. We do not have enough information to verify identification, but the black murky color of the water probably indicated an initial anoxic environment. By May 23rd, 2010, small non- filamentous Cyanobacteria were observed in SH4,

and by June 11th, 2010, with EC rising to 162 MS·cm⁻¹, the flora observed was mostly green-looking microbes. Despite the very high EC (159 mS·cm⁻¹) and the drop in pH to 6.5 (Fig. 3a, b) by August 25th 2010, filamentous Cyanobacteria succeeded to create mats (Fig. 10). A tentative identification is the halophytic *Phormidium hypolimneticum*. The mats did not exhibit the cable pattern of the ubiquitous haline species,

Microcoleus. The water in the sinkhole that used to be black now took to green. *Aphanothece* sp. was also seen, as well as some Protozoa. By October 2010, the mats were very intricate and the water appeared blue green. Two types of filamentous Cyanobacteria were seen in the mat, probably *Phormidium* and *Oscillatoria* spp., as well as solitary Cyanobacteria and some Protozoa. By January 2011, the pH rose and *Dunaliella* sp. was

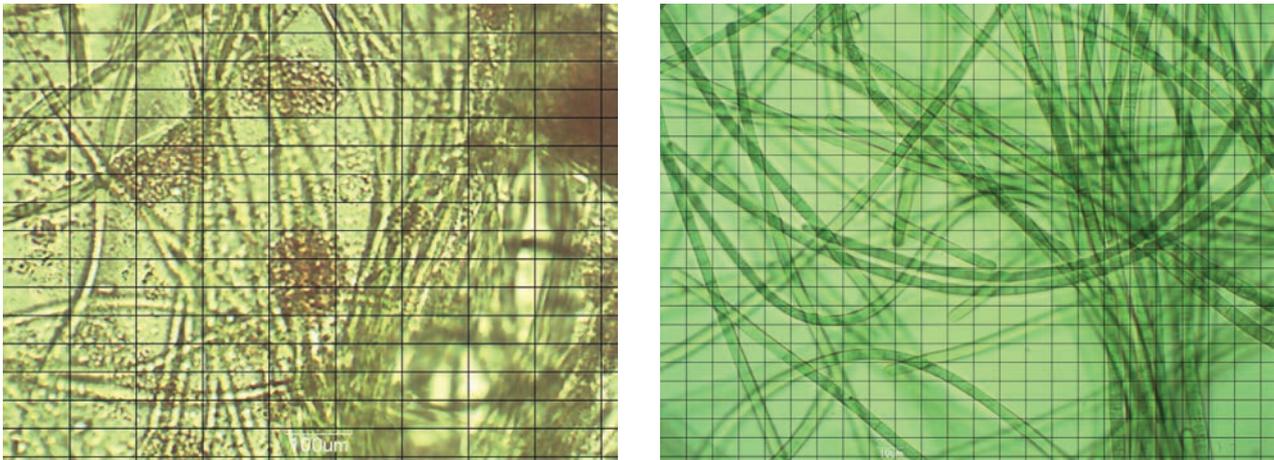


Figure 10: Cyanobacterial mats. Left: Mono-species Cyanobacterial mat (*aff. Phormidium hypolimneticum*) (SH4, 25.8.2010). Right: Bacterial aggregates inside Cyanobacterial mat (SH4, 17.10.2010)

observed. Interestingly, at that period a much more varied and complex biological picture emerged with the appearance of the filamentous Cyanobacteria mat and attached organisms.

3.3. Enrichment cultures

Notably, the enrichment conditions enhanced the proliferation of species that were not necessarily abundant, or even seen, in the original water samples. Thus, SH1 enriched for *Aphanothece/Euhalothece* and other small single-celled Cyanobacteria, and SH2 enriched for pico Cyanobacteria, 2-3 µm in diameter, and, following a 7-fold dilution, for a *Chlorella* sp. Diatoms and Protozoa thrived in enrichment cultures for a short while, but not in the long run. However, the ecological systems in the original samples left in the loosely closed collection bottles were found to still be alive with the same general community 12-18 months later: substantial mats, complete with diatoms, filamentous Cyanobacteria, shrimp, Nematoda and crabs proliferated in the SH1 collection bottles; *Aphanothece/Euhalothece* sp., *Synechococcus* sp. and diatoms thrived in SH2 bottles; dispersed Pennate diatoms were found in SH3 bottles, and complete Cyanobacterial mats with Protozoa and *Synechococcus* sp. were alive and prospering in a year-old SH4 collection bottle. Some of this data indicates that original cultures were under-sampled and some species that thrived overtime in the bottle conditions went undetected, as in the case of diatoms and *Synechococcus* sp. in SH2 from May 9th, 2010.

Overall, the temporal changes of flora and fauna in the sinkholes throughout the period of the research show an increase in the number of species over time, in particular during the spring and summer, with a vulnerability to changing external conditions that might jeopardize this process (Fig. 5).

3.4. Ecological dynamics

The four sinkholes, though very close to each other geographically, exhibited distinct patterns of ecological dynamics in constant interaction with an ever-changing physical/chemical environment. This is reflected in the low similarity indices between the sinkholes (Table 2), in the relative overall occurrence of floral and faunal phyla in the sinkholes (Fig. 4 a, b), and in the specific temporal dynamics depicted in figure 5 for each sinkhole. The higher similarity index between SH1 and SH3 reflects the dominance of mixed Diatomaceous/Cyanobacterial mats and the rich flora associated with them in those habitats (Fig. 3A), while the lower similarity index of SH4 with the other sinkholes reflects the dominance of lower life forms like bacteria and Cyanobacteria.

In SH1, a general pattern of gradual succession and species enrichment was observed throughout the sampling period, with some seasonal fluctuations. In contrast to SH4, a large array of species was recorded with representatives of many phyla and groups including higher Metazoa and several trophic levels.

Generally, four stages were observed: (1) a community consisting of Cyanobacteria, diatoms and Chlorophyta was already in existence when sampling commenced. 2) Rotifera and Protozoa introduction; (3) spring proliferation: a significant increase in the numbers of Cyanobacteria species, both single-celled and filamentous and Chlorophyta; introduction of Nematoda; (4) appearance of Arthropoda, both shrimps and crabs.

SH3 was initially quite similar to SH1, but physical/chemical changes in the sinkhole environment had major impact on the ecological community. By October 2010 a marked increase in salinity was observed, perhaps connected with physical changes that eventually led to the merge with SH2. As a result, the community experienced stress, and many species were missing or in a deteriorated state. By January 2011, the fusion with SH2 took place, accompanied with a further increase in salinity and resulting in a further diminution of species diversity and a regression to Bacteria/Archaea and unicellular Cyanobacterial domination. The physical-chemical changes were, most likely, linked to underground dynamics, as the sinkholes are connected underground through the water table. Sinkhole mergers are most probably due to the continued dissolution of the salt formation underground (Shoval, 2003).

Sinkhole 4, the most extreme environment studied, with EC never below 124 MS·cm⁻¹, is a classic example of succession. Four main stages were observed: (1) initially the milieu appeared black, probably indicating an anoxic layer, and the only life seen were bacterial colonies, most likely sulfide oxidizers; (2) appearance of single-celled pico - to nano - planktonic Cyanobacteria; (3) the creation of a mono-species filamentous Cyanobacterial mat; probably *Phormidium hypolimneticum* (4) introduction of a greater variety of prokaryotic and eukaryotic life in and around the mats. Due to the high salinity, and/or to other physical/chemical factors, this community did not develop into a complex society as seen in SH1 or SH3 during the sampling period, and no Metazoa were observed.

In contrast to sinkholes 1 and 3 that exhibited a multiplicity of taxa and taxonomic groups and could, at their best, be described as peak communities, SH2 featured a different pattern altogether. SH2 is characterized by single-celled algal/Cyanobacteria dominance rather than mat development. Originally, salinity was high and the pond was apparently controlled by *Dunaliella* sp. for about 6 months. Later, with rising temperatures and salinity dropping, *Aphanotece/Euhalothece* sp. predominated, with *Dunaliella* retreating in numbers but not disappearing. Similarly, Dor and Ehrlich (1987) found Cyanobacterial predominance over Chlorophyta in the summer (over 30°C) in artificial solar ponds. The prominent difference between SH2

and the other sinkholes studied was the lack of significant mat formation with resulting low species diversity. The difference between the sinkholes might be attributed to differences in mineral compositions. A strong smell of sulfur noticed in January 2010 in SH2 could indicate the existence of an anoxic layer at the time. There are accounts (Dr. Shiri Meshner, personal communication) of red *Dunaliella*-dominated sinkholes in the Ein Gedi area from 2013. It should be noted that the *Dunaliella* sp. in SH2 was not observed to be red.

3.5. Salinity and biodiversity

Salinity seems to be a main abiotic factor to affect biodiversity in the sinkholes. It is notable that when the salinity in SH1 and SH2 (and probably in SH3 as well) decreased towards spring 2010 there was a major increase in abundance of some species and phyla diversity in SH1 and SH3, especially in higher life forms, and a shift from *Dunaliella* dominance to *Aphanotece* dominance in SH2 (Fig. 4). By October 17th, 2010, EC had risen to 99 and 107 mS·cm⁻¹ in SH1 and SH3 respectively, adversely affecting species diversity, especially in SH3. SH1, having a more intricate and established mat community seemed to be less affected (Fig. 4). Even though Chlorophytes seemed to disappear from SH1 by October 17th, 2010, they reappeared in January 2011 while salinity stayed the same; *Aphanotece* also reappeared, but the Cyanobacterial element of the mat, mostly *Phormidium* spp. seemed to be missing. The increasing salinity of SH3 throughout the summer and fall may be linked not only to increased evaporation but also to physical changes leading to the eventual merge with SH2 observed in winter. Concomitantly with the gradual rise in salinity, the flora seemed to lose its diversity and richness, Chlorophyta and the large Nitzschoid diatoms disappeared and following the merging a predominance of Bacteria and Archea was observed, while some Pennate diatoms survived but seemed weakened. As to SH4, the most saline of the sinkholes, the species variety was indeed very limited initially and consisted of Archea, Bacteria, pico-Cyanobacteria and fungi all the way through June with EC levels not descending below 132 mS·cm⁻¹. By August 25th, 2010, despite persistent high salinity reaching 159 mS·cm⁻¹ and an actual drop in pH, a very developed Cyanobacterial mat dominated the water, changing the color from black to blue-green, and *Aphanotece* sp. appeared. (Figs. 2A, B, 4). By January 16th, 2011, with EC as high as 153 mS·cm⁻¹, *Dunaliella* sp. was present in the water.

The low similarity indices and the varying species composition might reflect different sinkhole ages. The developed communities in SH1 and SH3 probably reflect

sinkhole maturity, while the more limited biological ensembles in SH2 and SH4 could reflect a younger age or, alternately, more restrictive physical/chemical conditions. The sinkholes' distances from the sea were 17.23, 25.65, 19.89 and 7.22 m in ascending numerical order. Sinkholes 1 and 3, being at about the same distance from the sea have the highest similarity indices, while SH4, being the closest, has a low similarity index with all other sinkholes and was the most saline. It is, therefore, possible that its limited, more elementary flora reflects these conditions rather than its age. Established mats are a sign of a community's age. If the sinkhole colonization follows more or less the same pattern as the solar ponds (Dor et al., 1992), we might conclude that sinkholes 1 and 3 could be older than 5 years.

3.6. The biological mats

With the notable exception of SH2, the ecological communities, from a certain stage of development onwards, were based in and around mats. In general, three types of mats developed in the sinkholes: (1) filamentous Cyanobacterial-based, (2) diatom-based, and (3) mixed. The mat environment seemed to provide a relatively stable matrix around which affiliated species showed seasonal patterns, as well as a variety of niches for a plethora of flora and fauna to develop. It should be noted for clarification that the mats themselves were highly dynamic with all mat elements possessing motility, including diatoms and Cyanobacteria. When mats were not formed, as in SH2 and SH4 before the summer, the overall species diversity was more limited.

4. Discussion

In a thorough review about algal dispersal methods, Kristiansen (1996) describes four major vectors of algal distribution: water, air, organisms (internally or externally) and humans. The most efficient distribution is carried out by birds, but other agents such as dragonflies and damselflies can also transport viable spores and cysts over large distances; beetles have been shown to carry live algae over short distances from one water body to another; mammals can carry viable algae in their fur or in their intestines for several hours. Some diatoms have been shown to move actively from one pond to another along wet rock surfaces. Gislen (1948) wrote that "Wherever a biotope suitable for a certain micro-organism exists, that organism will appear there as soon as sufficient time has elapsed to allow it to be transported through the air and to settle in the locality." The species found in the sinkholes could have arrived there by underground waterways from the Judean Mountains, from the

Dead Sea through pore water in the sediments or by spray, as well as from other saline water bodies in the area, including other sinkholes, by any of the vectors mentioned. The newly found Dead Sea bottom organisms (Ionescu et al., 2004) are an interesting possible source as well. Exposed to abrupt changes in spring flow they are assumed to have a very wide range of salinity tolerance (Ibid), which makes them suitable candidates for colonizing the near shore sinkholes characterized with unstable conditions. Additionally, in a recent study (Hanegbi and Banet, 2013) a plethora of unicellular and filamentous Cyanobacteria, as well as some Chlorophyta were isolated from rocks in the Uvda Valley area, thus possibly expanding dispersal sources even beyond the watery domain.

The current study, although it reports similarities between the sinkhole communities and others in the area, leaves the question of the species origin and dispersal vectors at the level of conjecture.

In general, the flora of the sinkholes was similar to accounts of other saline bodies in Israel such as the saltern ponds in the Eilat area (Řeháková et al., 2009) and the Solar Lake in the Northern Sinai (Krumbein et al., 1975; Cohen et al., 1977). Similarity to the flora developed in the artificial solar ponds and studied by Dor and Ehrlich (1987) and Dor et al. (1992) was even greater. The newly established sinkholes were similar in the colonization process to the artificial solar ponds, as new ecological niches, and possessed a similar community of pioneering species. Prominent in both environments were species of *Phormidium*, *Ephanothece*, *Halospirulina*, *Nitzschia*, *Navicula* and *Amphora*. Chlorophyta were represented in the artificial solar ponds by *Euglena*, and in the sinkholes by *Dunaliella*. Mat formation in the solar ponds did not develop beyond the single layer even after 5 years of study. We did not observe complex mat layering either, but that, of course, could be the result of our method of sampling that involved mostly picking floating pieces of the mats, and our lack of data on the state of the benthos.

All sinkholes featured Cyanobacterial life, both unicellular and filamentous. The mat-forming *Phormidium* sp. (Fig. 11) could well be *P. hypolimneticum* (also called *Oscillatoria limnetica*), found by Cohen et al. (1975) to be capable of both oxygenic and anoxygenic sulfide-oxidizing photosynthesis in the dense hypolimnion of the Solar Pond in Sinai (74.1‰ salinity). The same species was found by Dor and Ehrlich (1987) in the artificial solar ponds in Ein Boqeq and Beit Haarava. *Phormidium*'s takeover of the biology of SH4 by June 2010 might indicate its previous existence in the sulfurized layer in the sediments as a sulfur oxidizer (indicated by the black water color and the smell of sulfur), as well as its

possible contribution as an oxygenic photosynthesizer in the eventual oxygenation of the pool.

Additionally, the free-floating Cyanobacteria tentatively defined as *Aphanothece/ Eualothece* sp., recorded in remarkable concentrations in SH2, and sporadically in SH1 and SH4, is similar or identical to a hyper-saline species found by Dor and Hornoff (1985 a,b) in a solar pond by the Northern Dead Sea and by Oren et al. (1995) in the Solar Lake, Sinai. The *Aphanothece/ Eualothece* sp. case might demonstrate the interrelationship between the sinkholes and other water bodies in the Dead Sea area. Several samples of the Heimar Reservoir near Neve Zohar taken by the authors showed a continuous mono-dominance of that species in the water column. The EC measured in the Heimar reservoir was 79 mS·cm⁻¹ (June 2nd, 2010). *Aphanothece halophytica*, also known as *Halothece*, is described by Seckbach and Oren (2007) as "the most widespread and best-known unicellular halophilic cyanobacterium". Exact taxonomy of that species is hard to come by due to the vast polymorphism in size, shape and color observed with changes in temperature and salinity (Dor and Ehrlich, 1987; Goh et al., 2010). An increased tolerance to temperature was observed with increased salinity, indicating a hardening effect allowing the organism to grow in water that is both more saline and hotter (Dor and Hornoff, 1985a). The cells of the *Aphanothece/ Eualothece* sp. were highly granulated (Fig. 8) and large for Cyanobacteria, probably due to accumulation of osmoregulants. Exopolysaccharides and Glycine betaine were indeed found to be produced by *Eualothece* strain HSC29 from the Australian Shark Bay stromatolites at high NaCl levels, and a considerable rise in cell size with salinity was also observed (Goh et al., 2010). Sucrose and glucosylglycerol were found to serve as osmo-regulants in *Aphanothece* species from freshwater and marine environments respectively (Reed et al., 1984).

Halospirulina tapeticola is another interesting Cyanobacterial species widely encountered in hyper-saline environments (Nubel et al., 2000; Seckbach and Oren, 2007). Nubel et al. have redefined highly halophytic *Spirulina* strains as a distinct genus: *Halospirulina*, with high salt and temperature tolerance and an incapacity to grow in fresh water. They also found, using 16S rRNA identification that halotolerant species capable of growing at 13% salinity from across the Atlantic, were closer genetically to each other than to the eurohaline species to freshwater species in their vicinity. They accumulate glycine betaine and glutamate betaine as osmolites. It seems plausible to assume that the *Halospirulina* species found in the Ein Gedi sinkholes would be in the same genetic cluster as the species

found in Guerrero Negro in Baja California and in La Trinitat salt works in Spain. Our species answers the requirements for the *Halospirulina* genus defined by Nubel et al. (2000), as it thrives in water temperature of 39°C in August, tolerates high salinities, uses gliding rotation for motility, lives in high sunlight, has a tightly closed helix shape and the helix width of up to 6µm. Tightly coiled *Spirulina* was also found in the Zara and Zerka Ma'in hot springs (Oren, 2008).

In the Chlorophyta category, *Dunaliella parva* is considered the quintessential alga of the Dead Sea itself. It had been shown to leave cysts in the sediments and to bloom only when the epilimnion mixes below specific gravity of 1.21, and when enough phosphate is available. The almost synchronous blooms across the lake support the theory of origination in benthic cysts (Oren et al., 1995; Oren, 1999). Similarly, *Dunaliella* in the form of sedimentary cysts is likely the source for sinkhole colonization, but other methods of transport are also possible, including from neighboring sinkholes. Most research on halophilic Chlorophyta was traditionally centered on *Dunaliella* sp. but halophilic strains of *Chlamidomonas* were collected in Chilca, Peru, and isolated by Yamada and Okamoto already in 1961. The strains grew in cultures as saline as 16% NaCl, with an optimum at about 10% and a minimum at 3%. Our samples show the existence of *Chlamidomonas* similes in SH1 and SH3 with varying numbers of flagella.

In the diatom category, we tentatively identify the sigmoid large mat-forming diatoms observed in SH1 and SH3 to be *Pleurosigma*, *Gyrosigma* or *Nitzschia sigma*. *Pleurosigma*, in particular, was shown to inhabit haline environments (Clavero et al., 2000, Řeháková et al., 2009). Furthermore, the three species of *Pleurosigma* found in evaporation and tidal pools in Guerrero Negro, Baja California Sur, were actually described as "the only true halophilic diatoms ever reported" and "incapable of growing in salinities less than 5%" (Clavero et al., 2000). *Pleurosigma* can reach up to 600 µm (UC Santa Cruz, date not given) and *Gyrosigma strigillis* was reported to measure 282 µm (Olenina et al., 2006). *Pleurosigma salinarium* was also found in saltern pond mats in the Eilat area (Řeháková et al., 2009). The very large non-sigmoid diatoms seen in the sinkholes are probably a *Nitzschia* sp., though due to their extraordinarily large size they might be a separate subspecies or strain endemic to the area. The majority of the large diatoms, and sometimes the smaller diatoms as well, were found in association with endophytic and epiphytic Cyanobacteria. The possibility of a mutualistic relationship based on nitrogen fixation is likely. Several studies report nitrogen fixation, probably executed by microbial endosymbionts, in floating diatomaceous mats in

the open ocean (Martinez et al., 1983; Villareal et al., 1999). Other Pennate diatoms observed were probably also similar to those found by those authors, mainly *Navicula* and *Nitzschia* spp., many of which were also found in association with Cyanobacteria.

In contrast to the research on solar pond colonization mentioned earlier (Dor and Ehrlich, 1987; Dor et al., 1992), we found higher trophic levels represented by both unicellular Protozoa and Metazoa such as crabs and *Artemia* shrimp in some of the sinkholes. Since the above authors attribute the lack of browsers to the special composition of Dead Sea brine, this difference must be explained by different water compositions. Regarding halophilic Nematoda, Pen-Muratove et al. (2010) reported several species of soil Nematoda in the hypersaline soils of the Arava desert. Pinder et al. (2003) recorded halophilic aquatic Nematoda (up to 30%) in the wheat belt region of Southwest Australia.

The miniature crabs might be the most interesting species to be found in the sinkholes. An Internet search did not find similar species in the records. The smallest crab species mentioned in literature were several millimeters long, visible to the naked eye. This could be an endemic species to the Dead Sea area. Further research into existing water bodies in the area and perhaps into possible underground connections further uphill is needed to determine the species distribution and living requirements. The living crabs observed in the collection bottles 12-18 months past sampling were in the same size category as those observed in the original sampling (up to 400 μm in width), a fact that supports the assumption that these are mature individuals of a miniature genetic line.

The survival of the ecological systems in the loosely-closed collection plastic bottles could be indicative of autonomous or nearly-autonomous systems, where even higher Metazoa like the crabs could endure over time. That suggests a very efficient circulation of nutrients and gases inside and around the mats. Several accounts report diatomaceous or mixed Cyanobacterial / Diatomaceous mat formation in highly saline water bodies (Krumbein et al., 1977; Oren et al., 1985; Waine, 2009). These might eventually result in stromatolite formation.

The current study was limited to those organisms easily observed by conventional light microscopy. It is reasonable to speculate, though, that Bacteria and Archaea would have been among the pioneering species to colonize the newly formed sinkholes, especially those with an anoxic layer and high salinities. Bacterial clusters were, indeed, observed consistently at SH4, especially at the earlier stages of succession when sulfur odor was easily detectable.

In order to better understand the effect of physical-chemical changes in the sinkhole milieu on the biota, chemical analysis of the water and soil using advanced methods such as HPLC-MS is called for. Additionally, DNA studies should be performed for definite taxonomic identifications and to establish phylogenetic trees. Employment of such techniques in conjunction with expanded sampling of the Dead Sea sinkholes would provide further insights into the unique and interesting characteristics of these pioneering communities.

In summary, the sinkhole environment provides an opportunity to study the process of colonization in new extreme environments. Different sinkholes, with varying physical-chemical conditions and at different stages of ecological development, demonstrated different patterns of colonization and succession. Salinity and oxygenation seemed to be crucial factors in determining species identity and diversity, but more physical-chemical data, such as ion and mineral composition of the water, O_2 and H_2S levels at different depths, sediment composition and temperature/salinity gradients, as well as primary productivity and photosynthesis data are needed to understand the biological processes, such as colonization, better. Microbial/algal mats seem to stabilize the ecological communities to some extent against changes in the dynamic physical environment, and to increase diversity. Communities of mats were also shown to be self-sufficient over an extended period of time. In contrast, sinkhole communities dominated by unicellular algae or Cyanobacteria appeared more vulnerable to physical/chemical changes in the environment. It is postulated that some species observed in the sinkholes may be endemic. Associations between diatoms, Cyanobacteria and microbes, both epiphytic and endophytic were observed and are assumed to help the partners adjust to the extreme environment. Further study of the sinkholes ecology could illuminate the complex interactions between the physical-chemical and biological environments as well as between different organisms in these newly-formed unique micro-habitats and contribute to the study of saline habitats and of halophiles.

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Appendix: A list of Video Hyperlinks

1. Diatom associations – epiphytes and endophytes, Halospirulina, Chlorophyta – <http://youtu.be/dWtEe8hPhW0>
2. Protozoan, Halospirulina, diatoms – <http://youtu.be/6sMbK3gQTIU>
3. Various filamentous Cyanobacteria, endobacteria? - <http://youtu.be/4xtCu0NY5zA>
4. Halospirulina in motion -<http://youtu.be/Oc5RTINIzza>
5. Crab feeding, Rotifers, Diatoms in motion, Ciliates - <http://youtu.be/gyxT2jui9vY>
6. Highly diverse peak community at SH1, including black bacteria clusters and endobacteria – http://youtu.be/P9_3ssysA0
7. Rotifera predating on huge diatoms – <http://youtu.be/11MeJ1nAmJI>
8. Diverse community at Sinkhole 1 – <http://youtu.be/6PPydFIEFbk>
9. Rotifer in diatom mat – <http://youtu.be/7pzbUjErKHI>
10. Chlorophytes, Halospirulina, diatoms – <http://youtu.be/MpWCxAgozpo>
11. Huge diatoms in associations, various filamentous Cyanobacteria – http://youtu.be/_czVkXGto64
12. Mini-crabs in mat – <http://youtu.be/7oLk2qDr3d8>
13. Miniature crabs – <http://youtu.be/oHcpYTMH01w>
14. Peak community at SH1: Crabs, shrimp, Nematoda, Cilata, Diatomea and Cyanobacteria <http://youtu.be/EpkV6AHtyNk>