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Characterization of a new parthenogenetic *Artemia* population from Thamaraikulam, India

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The brine shrimp *Artemia* (Crustacea, Anostraca) is a well-known organism used as live feed for finfish and shellfish larvae. Here we report on a new population of *Artemia,* identified in saltpans of Thamaraikulam, India. The characterization of this strain involved cyst, naupliar and adult biometrics, as well as carbohydrate, protein, lipid, ash and energy content estimations. The fatty acid profile of the cysts was also analysed. The cyst diameter, nauplius and adult length of this *Artemia* strain were 244.9 mm, 492.8 mm and 10.60 mm, respectively. Moreover, the optimal temperature and salinity conditions resulting in maximum survival were 22-30°C and 35 ppt, respectively. It is worth noting that this population can survive in a wide range of temperatures, but in a very narrow range of salinity. These data suggest that this new *Artemia* population is different from other studied parthenogenetic strains in terms of its biometrical characteristics and temperature and salinity tolerance.

Key words: *Artemia*, brine shrimp, parthenogenetic, characterization, India.

INTRODUCTION

Artemia is considered as an irreplaceable live feed for the larval rearing of most marine fish (Sorgeloos *et al.*, 2001) and shellfish species (Léger *et al.*, 1986; Bengtson *et al.*, 1991; Sorgeloos *et al.*, 1998). The demand for *Artemia* has increased with the rapid development of aquaculture industries. *Artemia* cysts harvested from the traditional harvesting ground of Great Salt Lake in Utah (USA) have met the global demand to a significant level. However, since the mid-nineties, there has been a decline in cyst yields due to the unfavorable biotope conditions (Stephens, 1998; Lavens & Sorgeloos, 2000), which created a bottleneck situation. This cyst shortage has intensified the exploitation of natural *Artemia* sites and it has triggered the need for alternative resources. Hence, the exploration of new *Artemia* habitats has been highly motivated in the recent years. As a result, several *Artemia* populations, especially in continental Asia, have been identified and characterized (Triantaphyllidis *et al.*, 1994, 1997a,b, 1998; Xin *et al.*, 1994; Pilla & Beardmore, 1994; Başbuğ & Demirkalp, 1997; Abatzopoulos *et al.,* 2002).

Natural populations of *Artemia* usually inhabit hypersaline environments (coastal or inland, chlorine, sulfate or carbonate-rich waters) and especially coastal salterns (man-made or man-managed solar saltworks) (Bowen *et al.*, 1985, 1988). Their geographical distribution is discontinuous and the populations are located in isolated biotopes of different climatic zones. The geographical separation of *Artemia* populations results in a number of different strains with various phenotypes and different biological, biochemical and physiological characteristics (Triantaphyllidis *et al.*, 1998). This may be due to the considerable ecological changes, which have occurred in those areas affecting population dynamics, as in Great Salt Lake (GSL) samples of 1966 and

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1976 (Stephens & Gillespie, 1976). Similarly, the nutritional quality of *Artemia* also varies among different strains from various locations (Bookhout & Costlow, 1970; Wickins, 1972; Beck *et al.*, 1980; Johns *et al.*, 1981; Klein-MacPhee *et al.*, 1982; Léger *et al.*, 1986) as well as among different batches of the same strain (Léger *et al.*, 1985). Therefore, the biometrical characteristics as well as the biochemical content of cysts, nauplii and adults are considered to be useful parameters for basic quality characterization of different *Artemia* batches. These studies could help identifing suitable strains for aquacultural uses, as the size and biochemical content of *Artemia* cysts and/or nauplii are important parameters for their potential application.

Artemia is generally considered as a summer species (Carpelan, 1957; Bayly, 1972; Stephens & Gillespie, 1972), inhabiting tropical as well as subtropical regions, where the water temperature and salinity vary extensively (Bowen *et al.*, 1978; Persoone & Sorgeloos, 1980). Different populations of *Artemia* have different levels of tolerance to water temperature and salinity. The variation in the tolerance levels may be further influenced by the genetic makeup of different strains (Bowen *et al.*, 1978; Abreu-Grobois & Beardmore, 1980, 1982; Abatzopoulos *et al.*, 2003; Baxevanis & Abatzopoulos, 2004). Several culture studies have revealed that the temperature and salinity conditions vary for different strains (Claus *et al.*, 1977; Vanhaecke *et al.*, 1984; Baxevanis *et al.*, 2004; El-Bermawi *et al.*, 2004). It has also been suggested that there is significant interaction between temperature and salinity for the survival of *Artemia* (Browne & Wanigasekera, 2000; Baxevanis & Abatzopoulos, 2004; Kappas *et al.,* 2004).

Approximately, eighteen *Artemia* sites have al-

TABLE 1. *Artemia* biotopes in India

Locality	Geographical co-ordinates	Mode of reproduction	Reference
Rajasthan			
Didwana	27°03'N-74°05'E	P*	Bhargava *et al.* (1987)
Sambhar Lake			Baid (1958)
Gujarat			
Balamba salterns	23°42'N-70°17'E	P	Royan *et al.* (1987)
Gulf of Kutch	23°20'N-71°00'E	P	Royan (1979)
Jamnagar	22°30'N-70°08'E		Royan (1979; 1980)
Mithapur	23°00'N-70°10'E	P	Royan (1979; 1980)
Mumbai			
Bahinder	18°55'N-72°50'E		Dwivedi *et al.* (1980)
Bhayander	18°55'N-72°50'E	P	Kulkarni (1953); Bohra (1980)
Vadala	18°55'N-72°50'E		Royan *et al.* (1978)
Chennai			
Kelambakkam	13°05'N-79°07'E		Kulasekarapandian *et al.* (1992)
Vedaranyam	10°01'N-79°50'E		Basil *et al.* (1987)
Tuticorin			
Harbour	8°50'N-78°08'E		Ramamoorthi & Thangaraj (1980)
Karsewar Island	8°50'N-78°10'E		Achari (1971)
Pattanamaruthur	8°55'N-78°08'E		Ramamoorthi & Thangaraj (1980)
Saltwater springs	8°50'N-78°08'E	P	Royan *et al.* (1970; 1978); Lal Mohan (1980); Ramamoorthi & Thangaraj (1980)
Spic Nagar	8°50'N-78°08'E		Ramamoorthi & Thangaraj (1980)
Thirespuram	8°50'N-78°08'E		Ramamoorthi & Thangaraj (1980)
Veppalodai	8°59'N-78°08'E		Royan *et al.* (1970)
Kanuakumari			
Thamaraikulam	8°04'N-77°68'E	P	Present study

* Parthenogenetic

ready been identified in India, with all strains being parthenogenetic (Table 1). As tolerance to temperature and salinity is inter-related for aquatic invertebrates (Kinne, 1970), a factorial design of experiments involving 25 combinations of five temperatures and five salinities was used in the present study. The aim was to characterize a new *Artemia* population for Thamaraikulam saltpans (India) for its tolerance to water temperature and salinity and to determine the optimum conditions for maximum survival. Its biometric and biochemical characteristics have also been examined.

MATERIALS AND METHODS

Physico-chemical parameters

The air and water temperature was recorded using a thermometer (Hermes, India; sensitivity ± 1°C). Salinity refractometer (New S-100, Tanaka Sanjiro Co., Ltd., Japan; sensitivity ± 1 ppt), pH meter (Elico, India) and hydrometer (Barigo, Germany, sensitivity 1%) were used to record the salinity, pH and humidity, respectively.

Biometrics of cysts and nauplii

Cysts collected from the condensers of Thamaraikulam saltworks were cleaned and dried. The diameter of hydrated cysts (n = 100) was measured under a microscope equipped with a calibrated micrometer eyepiece, following Vanhaecke & Sorgeloos (1980). The chorion thickness was also calculated after decapsulation (Sorgeloos *et al.*, 1986).

To analyze the biometrical characteristics of nauplii, cysts were hatched by incubating them in natural seawater (35 ppt) at 29 ± 1°C with continuous illumination of 1000 lux. The length of nauplii (n = 100) was measured under a microscope. Dry weight analyses of cysts and nauplii were carried out following Royan *et al.* (1987).

Morphometry and weight of adult Artemia

Artemia (25 adults) collected from a culture tank were treated with 2% Lugol solution and used for length measurements. Length of abdomen, length of furca, width of brood pouch, distance between eyes, diameter of eye, length of first antenna, width of head and total body length (from the tip of head to the end of caudal furca) of adult *Artemia* were also measured under a microscope using a micrometer (Amat, 1980; Başbuğ & Demirkalp, 1997). The weight of the animals was estimated using a monopan balance (SICO, SB3/200, India; sensitivity ± 0.1 mg).

Biochemical analyses

Carbohydrate, protein, lipid and ash content of cysts, nauplii and adult *Artemia* (wild) were estimated following Roe (1955), Lowry *et al.* (1951), Barnes & Blackstock (1973) and Paine (1964), respectively. The fatty acid profile of cysts was analyzed after Miller & Berget (1985). The total lipid isolated from the cysts was processed in four steps: saponification, methylation, extraction and base wash. A diethyl glycerol succinate (DEGS) column was installed in a 5890A gas chromatographer (Hewlett Packard) and used for the analysis. The operating conditions were as follows. Carrier gas, nitrogen: 30 ml min^{-1}, oven temperature: 180°C (isothermal), injection port temperature: 200°C, detector: flame ionization detector (FID), FID temperature: 230°C. The energy content was also estimated in a Parr 1421 semi microbomb calorimeter (Parr instrument Co., Moline, USA).

Culture media with different salinities

Culture media of different salinities were prepared by adding the appropriate amount of coarse salt to filtered seawater. The pH was maintained at 7.9 (using $NaHCO_3$ and/or Na_2CO_3) and it was never allowed to drop below this level, in order to avoid negative effects on the survival of larvae (Provasoli & D'Agostino, 1969).

Temperature and salinity tolerance test

The salt- and thermo-tolerance of this *Artemia* strain were determined at five temperatures (18, 22, 26, 30 and 34°C) and five salinities (5, 15, 35, 70 and 120 ppt). The tests were carried out in glass jars (200 ml capacity), each containing 10 nauplii in 100 ml medium. Mild aeration was provided. The jars were covered with perforated Petri dish to minimize evaporation. The nauplii (instar-I) were acclimatized initially for a period of 2 h, to prevent mortality due to temperature or salinity shock. The temperature and salinity were kept constant within ± 0.2°C and ± 1 ppt, respectively. The photoperiod was maintained as L:D = 16:8. Three replicates were scored for each combination. Survival was recorded on a daily basis (morning and evening) for a 9-day period. This ex-

perimental period was chosen because it has been previously suggested that a period of 9 days is appropriate for the determination of the range of temperature and salinity tolerance, as the larvae may reach the pre-adult stage and further mortality may be negligible beyond this period (Vanhaecke *et al.*, 1984). The data were subjected to two-way analysis of variance (ANOVA) as described by Zar (1974).

RESULTS

A new *Artemia* population consisted of only females has been identified in the Thamaraikulam saltpans, located at 8° 04′ N - 77° 68′ E (Fig. 1). It has been designated as parthenogenetic *Artemia* KKT1 (Kanyakumari-Thamaraikulam, batch 1). *Artemia* KKT1 has been found in the condenser of the saltworks. It is usually more abundant at the surface during early morning and late evening hours while moving to the bottom during daytime. Table 2 describes the micro-algae found in the water, which are probably the most important food source for *Artemia* KKT1.

Abiotic parameters of the habitat

The air temperature and humidity were 28.5 ± 4.20°C and 72.33 ± 2.19%, respectively. The water temperature, salinity and pH were 30.6 ± 3.58°C, 96 ± 22.84 ppt and 6.51 ± 0.15, respectively.

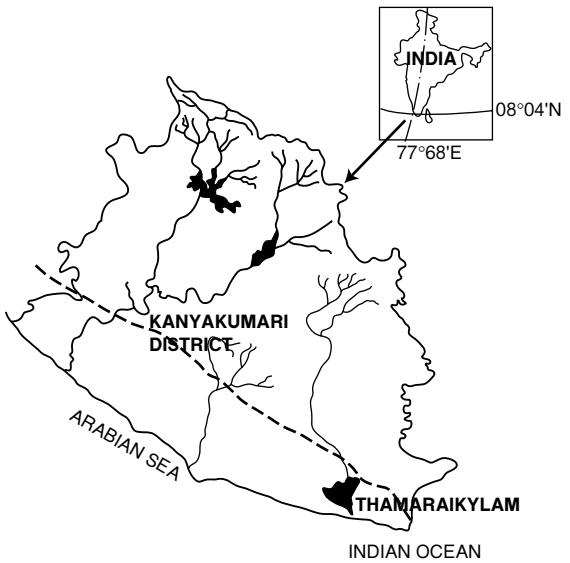

FIG. 1. Map showing the *Artemia* study site of Thamaraikulam in Kanyakumari District of India.

Population density and biomass of Artemia *KKT1*

The population density of *Artemia* KKT1 varied widely, i.e. from 4 to 650 individuals per litre. The biomass also fluctuated accordingly, i.e. from 0.002 to 1.32 g l^{-1}.

Biometrics of cysts, nauplii and adults of Artemia *KKT1*

The diameter of hydrated and decapsulated cysts and the individual dry weight are presented in Table 3. Similarly, the length and dry weight of nauplii are also given in Table 3. The size frequency distributions of cyst (diameter) and nauplii (length) are shown in Figs 2 and 3, respectively.

The morphometric parameters, such as total body length, length of abdomen, furca and first antenna, width of brood pouch and head, distance between eyes and diameter of eye are shown in Table 4. The length and weight relationship, from nauplius to adult stage was studied (data not shown). The correlation obtained for the relationship between length and weight was highly significant ($\log y = -1.6021 + 2.4135 \log x$; $r = > 0.9$; $p < 0.05$; $n = 14$).

Biochemical analyses

The biochemical contents of protein, carbohydrate, lipid, ash and the energy content of cysts, nauplii and adults of *Artemia* KKT1 were determined (Table 5). The fatty acid profile of cysts revealed that oleic acid

TABLE 2. Microalgae identified from the common condenser of Thamaraikulam saltpans

Strain	Size (μm)	Abundance
Cyanophyceae		
Oscillatoria	30	Major
Nostoc	30	Minor
Lyngbya	–	Minor
Chroococcus	–	Minor
Bacillariophyceae		
Pleurosigma	–	Major
Achnanthes	30	Minor
Gyrosigma	60	Major
Navicula	30	Minor
Pinnularia	30	Minor
Diatoma		Minor
Chlorophyceae		
Cladophora	–	Minor

– : not determined

TABLE 3. Biometrics of *Artemia* KKT1 cysts and nauplii

Characteristics	Range	Mean	SD
Diameter of hydrated cysts (μm)	217-289	244.9	7.40
Diameter of hydrated decapsulated cysts (μm)	196-267	228.1	8.40
Chorion thickness (μm)	8-10	8.4	0.61
Nauplii length (μm)	409-539	492.8	21.43
Individual dry weight (μg)	–	3.5	0.28
Individual naupliar dry weight (μg)	–	2.87	0.16

TABLE 4. Morphometric characters of adult *Artemia* KKT1. All measurements are in mm

Morphometric characters	Mean ± SD	Range
Total length	10.600 ± 1.076	9.000 –12.500
Abdominal length	4.250 ± 0.567	3.000 – 5.300
Width of ovisac	1.390 ± 0.200	0.750 – 2.000
Length of furca	0.556 ± 0.209	0.300 – 1.000
Distance between eyes	1.260 ± 0.143	0.900 – 1.500
Diameter of eye	0.330 ± 0.165	0.060 – 0.600
Width of head	0.760 ± 0.228	0.380 – 1.050
Length of first antenna	1.160 ± 0.182	0.730 – 1.620

TABLE 5. Biochemical composition of *Artemia* KKT1

	Protein (%)	Carbohydrate (%)	Lipids (%)	Ash (%)	Energy (KJ g^{-1})
Cysts	48.0 ± 0.91	27.8 ± 1.15	15.6 ± 0.35	8.6 ± 0.8	19.65 ± 1.361
Nauplii	50.6 ± 0.56	25.7 ± 1.32	14.2 ± 0.90	9.4 ± 0.73	18.97 ± 1.255
Adults	58.8 ± 0.66	18.9 ± 1.53	18.1 ± 0.55	4.2 ± 0.21	20.79 ± 1.932

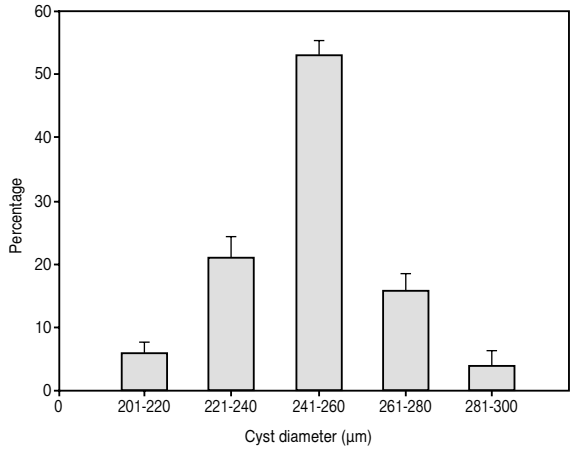

FIG. 2. Size frequency distribution of *Artemia* KKT1 cysts.

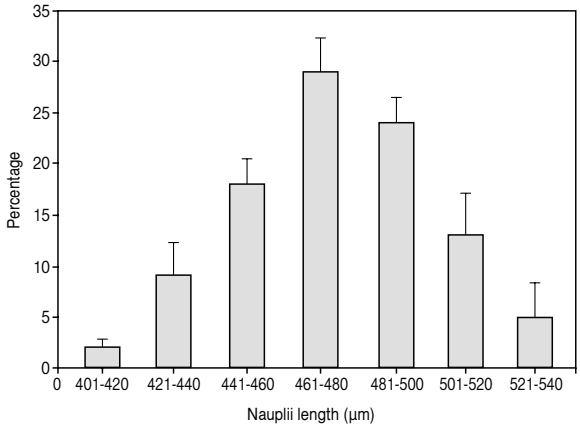

FIG. 3. Size frequency distribution of *Artemia* KKT1 nauplii.

(18:1n9) was the most abundant fatty acid (29.18 mg 100 g^{-1} of lipid). The saturated and monoene Fatty Acid Methyl Esters (FAMEs) such as 16:0, 16:n7 and 18:1n9 were 53.54 mg 100 g^{-1} of lipid. The other major fatty acid, linolenic acid (18:3n3) was estimated at 4.73 mg 100 g^{-1} of lipid.

Temperature and salinity tolerance of Artemia *KKT1*

Figure 4 illustrates the survival of *Artemia* KKT1 reared at different temperatures (18, 22, 26, 30 and 34°C) and salinities (5, 15, 35, 70 and 120 ppt). At the 18°C-5 ppt combination, all tested *Artemia* died within 5 days whereas *Artemia* reared at 15, 70 and

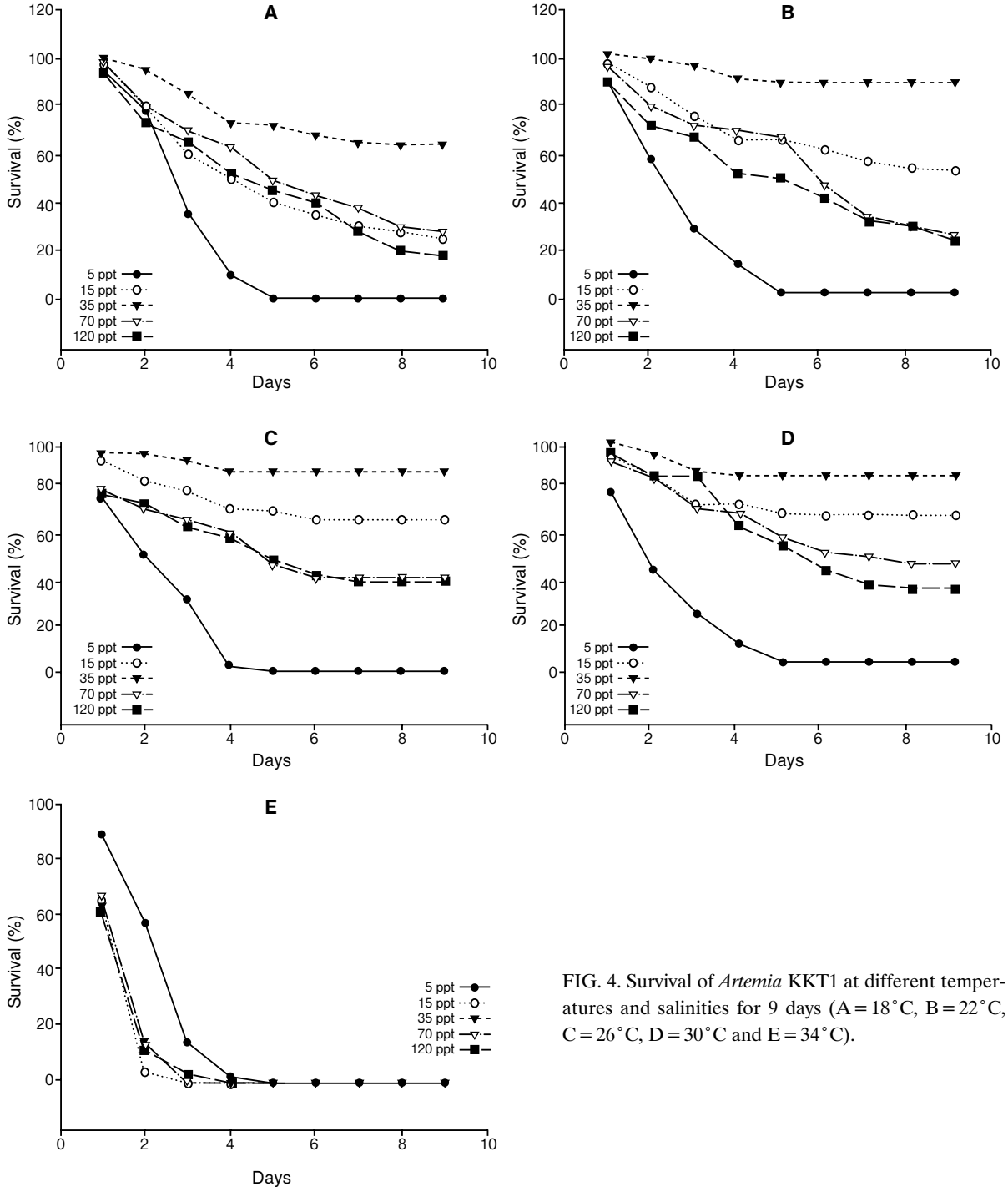

FIG. 4. Survival of *Artemia* KKT1 at different temperatures and salinities for 9 days (A = 18°C, B = 22°C, C = 26°C, D = 30°C and E = 34°C).

TABLE 6. Two-way analysis of variance (ANOVA) for the influence of temperature and salinity on the survival of *Artemia* KKT1

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	Variance ratio	*p* value
Temperature	4	7636.84	1840.96	8.23	<0.001
Salinity	4	11229.44	2807.36	12.55	<0.001
Error	16	3578.56	223.66	–	–
Total	24	22171.84	–	–	–

$p < 0.05$ is statistically significant

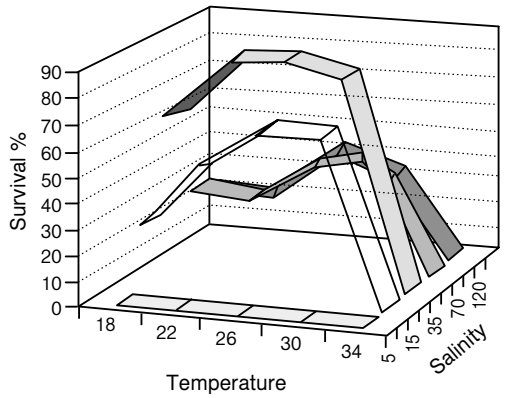

FIG. 5. Schematic representation of *Artemia* KKT1 survival at different temperatures and salinities after 9 days of culturing.

120 ppt showed survival between 18 and 30%. Individuals reared at 35 ppt exhibited maximum survival (64%) after 9 days of the experimental period. At 22˚C, no survival was found on the fifth day, in salinity 5 ppt. At 35 ppt, over 85% of the animals survived on ninth day, whereas at 15 ppt 51% survival was recorded. For the individuals reared at 70 and 120 ppt, the survival was around 20%. For individuals reared at 26˚C, the survival was almost 0% at 5 ppt on fifth day. The survival of *Artemia* increased to >40% at 70 and 120 ppt, to >60% at 15 ppt and to a maximum of 90% at 35 ppt salinity. The trend at 30˚C at the given salinities was more or less similar to that of 26˚C. At 34˚C, all the individuals reared at 15, 35 and 70 ppt, died within 2 days. At the two extreme salinities (5 and 120 ppt), the animals survived up to 5 and 3 days, respectively.

The data obtained from this experiment were treated statistically and it was revealed that both temperature and salinity influenced significantly the survival of *Artemia* KKT1. Salinity bore a stronger effect, [$F_{4,16} = 12.55$] than temperature [$F_{4,16} = 8.23$] (Table 6). These data suggest that the optimal temperature and salinity for maximum survival of *Artemia* KKT1 are 22-30˚C and 35 ppt, respectively (Fig. 5).

DISCUSSION

Investigating new *Artemia* populations and their biometrics as well as biochemical characteristics are suggested to be more intensified nowadays for meeting the ever-increasing demand for *Artemia* (Triantaphyllidis *et al.*, 1998). Such efforts have been reported by several authors (Triantaphyllidis *et al.*, 1994; Xin *et al.*, 1994; Pilla & Beardmore, 1994; Triantaphyllidis *et al.*, 1997a,b; Zuniga *et al.*, 1999). In this study, we present the characterization of a new *Artemia* population from Thamaraikulam, India. Cyst diameter is generally considered to be strain-specific (Vanhaecke & Sorgeloos, 1980) and is therefore a practical tool, although fairly weak, for strain identification. The cyst diameter of parthenogenetic *Artemia* KKT1 is 244.9 μm, which is closer to that of the bisexual strains, GSL (244.2 μm) and Canadian (240 μm). However, there is significant variation, similar to those of Citros and M. Embolon strains (Abatzopoulos *et al.*, 1989), in the size of KKT1 *Artemia* cysts.

The micro-algae content in *Artemia* ponds was determined (Table 2). It has been demonstrated that algae composition not only influences growth and reproduction, but also has a considerable effect on the nutritional value of biomass and cysts (e.g. fatty acid composition) (Evjemo & Olsen, 1999; Fabregas *et al.*, 2001). Moreover, the benthic and/or filamentous algae are unsuitable for *Artemia* as food, therefore, development of these algae must be prevented. Otherwise, the filamentous algae blooming in the ponds, spread quickly and finally interfere with cyst collection. It has been reported that a group of algae, which produces toxins in certain ecosystems may not produce toxins in other ecosystems, depending on the prevailing environmental conditions (Phlips,

2001). Also, some species of *Oscillatoria* (which was abundant in the study site) have been shown to be toxic to *Artemia* (Reinikainen *et al.*, 1995; Smith, 1996). Therefore, the role of algae on the growth and biology of *Artemia* deserves further evaluation.

The size of *Artemia* nauplii of SFB, PHIL and ARG commercial strains has been shown to be suitable for shrimp hatchery, as the shrimp larva size is also small (Léger *et al.*, 1986). Although *Artemia* KKT1 nauplii may be marginally suitable for shrimp hatchery because of their larger size, they can be used for finfish hatcheries. Moreover, the naupliar size of KKT1 is suitable for bioencapsulation to deliver hormones aiming at induction of ovulation (Burton *et al.*, 1998), drugs (Touraki *et al.*, 1999; Majack *et al.*, 2000) and oral vaccination to economically valuable finfish larvae (e.g. gilthead sea bream *Sparus aurata,* European sea bass *Dicentrarchus labrax* and Atlantic cod *Gadus morhua*).

According to adult morphometric characters, KKT1 population was found to be closer to Egyptian asexual populations (El-Bermawi *et al*., 2004) due to similarities in all studied characters barring the length of the furca, which, in fact, is one of the largest recorded in *Artemia* genus (Triantaphyllidis *et al*., 1997a; El-Bermawi *et al*., 2004). To some extent, similarities have also been observed with Chinese *Artemia* strains (Triantaphyllidis *et al*., 1997b).

The biochemical composition in protein, carbohydrate and lipid of *Artemia* nauplii generally varies considerably: 37-71%, 11-23% and 12-30%, respectively (for comparison purposes see Bengtson *et al.*, 1991; Evjemo & Olsen, 1997). The ash content also varies form 4 to 21% (Léger *et al.*, 1986). The levels of protein, carbohydrate, lipid and ash content of adult *Artemia* were reported to be 50-69%, 9-17%, 2 -19% and 9-29%, respectively (Léger *et al.*, 1986). Except for adult ash content, the other biochemical contents of the KKT1 strain fall within the range of the reported values. Sandoval *et al.* (1993) revealed that the protein content of *A. fransciscana* cysts was 41.4-53.3%, and that of *Artemia* KKT1 strain was found to be 48%. The carbohydrate content of cysts was found to be higher than that of adults (Table 5) due to the high content in trehalose and glycerol of the former. Regarding the fatty acid profile, *Artemia* KKT1 is lacking the important highly unsaturated fatty acids (HUFA), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This is also the case in many commercially available *Artemia* strains (Sorgeloos *et al.*, 1991). Apart from these two essential fatty acids, it has been shown that arachidonic acid (ARA) also plays a significant role on the fish larval growth and pigmentation (Castell *et al.*, 1994; Estevez *et al.*, 1997; Koven *et al.*, 2000). However, these deficiencies could be remedied by enrichment or boosting procedures in which different kinds of emulsified HUFAs are incorporated into the *Artemia* nauplii in order to enhance their nutritional value (Sorgeloos *et al.*, 2001).

Temperature and salinity tolerance data suggest that KKT1 strain can tolerate a wide range of temperatures from 22 to 30˚C (Fig. 5), which is also reported for other *Artemia* populations (Barata *et al.*, 1996a). However, the salinity tolerance is highly limited to 35 ppt. Although temperature as well as salinity influence the survival of *Artemia* significantly, the effect of salinity was more pronounced compared to that of temperature (Table 6). Barata *et al.* (1996b) demonstrated that parthenogenetic strains are more tolerant to high temperatures (24-30˚C) as they showed high survival and population growth compared with sexual strains. However, contradicting data with narrow tolerance range were reported for a parthenogenetic strain (Browne & Wanigasekera, 2000), indicating that temperature-salinity tolerance limits vary for different strains and also contribute to strain characterization. India hosts a great number of *Artemia* sites (few of them are presented in Table 1). Unfortunately, information on these sites is very restricted, if any. Considerable efforts have to be invested to describe *Artemia* biodiversity in this vast country. In conclusion, the present temperature-salinity tolerance study provides useful information regarding the choice of suitable *Artemia* strains to be introduced to salterns with a given temperature-salinity regime and it also supplies practical guidelines with optimal conditions for easy determination of maximum *Artemia* productivity.

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