Studies on the Bacterial Causes and Problems Facing Artificial Spawning of *Artemia*

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Abstract
Four types of *Artemia* cysts were reared in the experiment for investigating bacterial causes and problems facing artificial spawning of *Artemia* in the way of obtaining *Artemia* population. It was established close recycling water rearing unite consists of ten aquaria as system for culturing. Rice bran suspension, yeast suspension and soy bean milk suspension were examined as feed for rearing. Soy bean milk suspension was the fastest metamorphosis and the highest cysts production. Bacteriological examination of sea water revealed at of a 260-280 C.f.u. / ml in the dilution of 1/100000 equal to $2.6\text{-}2.8x10^7$ and that was one of causes which may affect hatchability of the cyst. Using of probiotics was better than using of antibiotics in culturing of *Artemia* for disease control.

Key words: *Artemia*, infection and probiotics

Introduction

Artemia represent one of specific feed source for the early hatched fish fry that mainly related to its highly nutrients content of animal origin facing the most requirement of fry at this stage ***(Bardach et al., 1972).***

Artemia as the other aquatics can be affected by pathogens that present in the surrounded environment which causes economic losses varied from mortality of the adult stages to losses in the cyst production ***(Lavens and Sorgeloos, 2000).***

Bacterial diseases are the most causative agents of *Artemia* illness that creating infection vary from internal or external disorders to heavy mortalities in both wild and cultured *Artemia*. The main role of the invaded bacteria graduated from as first invasive microorganism responsible about the disease to secondary bacterial invasive causing development of the occurred disease ***(Abatzopolulos et al., 2010).***

Vibrosis is a bacterial disease occurred in marine and fresh water fishes caused by many strains of *Vibrio* represented in 3 forms acute, subacute and chronic. The most common are *V. anguilluarium* and *V. parahaemolyticus* which appearing in many affected marine and brackish water fish than fresh water fish and consider as

mainly *Artemia* pathogen ***(Hubbert, 1989).***

The need for *Artemia* cysts for hatcheries has been increased with the time of development of commercial marine fish culture, and the world need more than hundreds metric tons per year. The most famous localities in the *Artemia* cysts production were Great Salt Lake (U.S.A) which has been the premier suppliers to the world aquaculture market and the subject of Lavalduc, France then numerous speculations regarding grow in that aquaculture industry ***(Bassey, 2011).***

Egypt goes in the way for developing marine aquaculture systems that need *Artemia* as a food at fry stages; at time import of *Artemia* elevates the production coast. So, the aims of this work studying the disease and problems are facing artificial spawning and rearing of *Artemia* to produce native brand of *Artemia* cysts.

Material and methods

Culture system design

The used culture system (Fig. 1), consists of recycling water system of ten culture aquaria, distribution unit for the culture water with a reservoir, tank, disinfection part and supply pipe, outlet system with cyst filter for each aquaria and a precipitated tank. The aquaria made from transparent glass with hold volume of 100 liters each. Aquaria were arranged in separate groups. Inlet water is run up from a $3m^3$ tank.

Practical water source of the culture system

Two types of water were used during experiment period; natural sea water was obtained from Suez Canal, Ismailia governorate and artificial sea water made by addition of sodium chloride as advised by ***Sorgeloos et al. (1983).*** Salinity was adjusted to 35‰. Water was changed in rate of 1/10 every day. The pH range 7·2 - 7.8. *Artemia* was maintained for two months.

Artemia cysts

Four types of *Artemia* cysts were reared in the experiment for obtaining *Artemia populations* E1, E2 obtained from El-max Company, F1 from Lavalduc, France and F2 from Great Salt Lake, Utah, USA.

Floating test

Four types of *Artemia* cysts were examined against normal saline for detected purity degree ***(Lavens and Sorgeloos 2000).***

Decapsulation of *Artemia* cysts

Hypochlorite solution used as primary step for removing the external layer of a cyst. These cysts were hydrated in funnel shape container with artificial sea water and strong air flow from the bottom of the container ***(Anderson et al., 1970).***

Production yields

Production yields were estimated for the four types of cultured *Artemia* according to ***Provasoli and Pintner (1980).***

Testing of feed types

It was conducted with three trials; rice bran suspension, yeast suspension, and soy bean milk suspension. The three different types of feeds were compared with two replicates for each *Artemia* types. The feeding frequency was arranged as 2 times per day with 0.2 ml/L, mainly depended on water transparency. The growth rate was recorded for one month and the weight of the produced cysts was collected for two months.

Bacteriological examination of water

It was done according to ***Austin and Austin (2007)*** as following:

A) Total bacterial count

Bacterial colony counting of water sample collected from the rearing system was done during the time of experiment.

B) Isolation and identification

Samples for bacteriological examination were taken under aseptic conditions. Water samples were inoculated into Tryptic soy broth (T.S) and brain heart infusion (BHI) agar, then incubated at 25 – 29°C for 48 hr. The media were supplemented with 2% sodium chloride. The obtained colonies were purified and identified using routine examination of the morphology and biochemical reaction.

Microbial examination of *Artemia*

A) Examination of *Artemia* cyst:

Artemia cyst (0.5 g) was soaked in sterile T.S Broth for 24 and 72 h. at 28^{o} C and 15^{o} C respectively. The results of growth were recorded.

B) Examination of the nauplii:

By sterile mesh and sterile forceps a small amount of hatched nauplii were picked up and inoculated into T.S broth and steps previously mentioned before.

Antibiotic sensitivity test

The identified bacterium isolates were examined against 5 antibiotic discs (ampicillin, chloramphenicol, cibrofloxacin, florfenicol and tetracycline) using disk diffusion methods on Muller's Hinton agar medium (Oxoid), interpretations zones of inhibition were measured according to ***Buchanan and Gibsons, 1974.***

Microscopical examination of water

20ml of water sample from each aquarium were taken. The sample content was concentrated by centrifuged at 5000 rpm /15min. the sediment was separated and examined under microscope ***(Austin and Austin 2007).***

Trials for bacterial control

Three different trials of bacterial control techniques (Table 1) were tested with two replicates for each. For this, *Artemia* nauplii (7000 pcs/L) were stocked in 50 L of artificial sea water. The survival rate was recorded for one month.

Effect of variable sodium chloride concentration on *Artemia* survival

Adult stages of the *Artemia* were tested against different levels of sodium chloride concentration (from 35‰ up to 65‰) in the rearing water ***(Persoone and Sorgeloos 1980).***

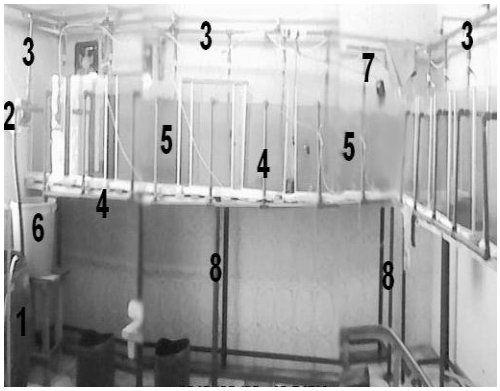

Fig 1: Culture system
1. Water store 2. Water pumps 3.Water pipes
4. Water siphons 5. Stone and sand filters 6.Glass aquaria
7. Air pumps 8. Heaters

Table (1): Trials of diseases control

Methods	Techniques 1	Techniques 2	Techniques 3
Types of treatment	control	probiotics	antibiotics
Forms of use	-	*Lactobacillus*	tetracycline hydrochlori
Dosage	-	10 ppm	2 ppm
Frequency (per	-	One time daily	2 times / day

Results and Discussion

In the experiment *Artemia* populations from El-MAX Company E1, E2, Lavalduc and Great Salt Lake (F1, F2) used in the present study for two months. Examination of used types against normal saline indicated more purity in F (1) and F (2) types than E1and E2 (Photo 2). Production yields optimized to maximize yields with E1, E2, F1and F2 yields were 0.3, 0.3, 0.5 and 0.4 g respectively cysts/ aquarium in the day.

The effect of the nutrition on the reproduction of the *Artemia* play the basic role in this activity in similarity to the other species. In addition to the amount of the produced larvae can be controlled by many types of diets used in the *Artemia* rearing that based on the results cited by ***Provasoli and Pintner (1980)*** that lipids are essential for the continued fertility of *Artemia*. In the present study *Artemia* were cultured depending on the effects of variable feeds (energy and protein source). It was conducted with three trials; rice bran suspension, yeast suspension and soy bean milk suspension. After two month, it was found that the soy bean milk suspension was the fastest metamorphosis and the highest cysts production (Fig.3 and table 3).

The bacteriological examination of sea water revealed at 260-280 C.f.u. / ml in the dilution of 1/100000 i.e. equal to $2.6\text{-}2.8\text{x}10^{7}$ and that was one of causes affecting hatchability of the cyst and agrees with 10^{6} C.f.u. ml^{-1} in water that obtained by ***(Austin and Allen, 1982).***

The microbial examination of cyst not revealed any bacterial or fungal growth although the incubation period extended to more than 72 h. and this agree with that obtained by ***Angelidus and Baudin (1990),*** they recorded permissive level of bacteria in the *Artemia* cysts should

be less than one bacterium per cyst, after hatching, bacteriological examination of the nuplii hatched and reared in sea water resulted in isolation of two types of vibrios; *vibrio alginolyticus* (isolate A) and *vibrio parahemolyticus* (isolate B) Table (4). Bacterial count (mainly vibrios) was 10^3 C.f.u. / nauplius and 10^6 –10^8 C.f.u./ ml in homogenate from *Artemia*. The results were agree with that obtained by ***Frerich, and Hendrie (1985)*** who suggests that *Vibrio* spp. founded in sea water can be colonized on *Artemia* cyst.

Antimicrobial sensitivity test revealed both isolates resist Ampicillin and sensitive to Cibrofloxacin, Tetracycline and Flofenicol as shown in Fig. (4) and table 5

The bacterial control techniques were measured according to survival rate (Table 6 and Fig.5). The results revealed that probiotics was better than using of antibiotics for culturing of *Artemia*. The result was supported by ***(San et al., 2008)*** who recorded using of probiotics were only chosen for *Artemia* rearing since the antibiotics affects adversely on the benefits and commensally bacteria in the surrounding aquaria.

Macroscopical examination of natural sea water showed heavy algal growth that leads to change the normal water color. So, different water samples were collected from the aquaria and examined microscopically to show heavy growth of many algal species specially *Chroococcus minutus* and *Microcystis* (Cyanobacteria) (Fig. 6). These algae are acting as a problem in the rearing system ***(San et al., 2008)*** since they produce cyanotoxins affecting on the viability of Artemia stages. The trails for using of chemicals agents for cleaning the system during the rearing process were failed and so, we used artificial sea water in the experiment.

In natural environments impacts of salinity on *Artemia* populations were mostly important factors influencing *Artemia* population growth, percent of cyst hatching, survival degree and methods of reproduction ***(Browne and Wanigasekera 2000).*** In the present work *Artemia* were survived in artificial sea water that was prepared in the wet laboratory by using raw sodium chloride not containing iodine. After acclimatization, some individuals were survived indefinitely in different dilutions varying from 35‰ up to 65‰. Also, *Artemia* survive for several days in isotonic sodium chloride solutions. The results were supported by ***Vos, (1979)*** who cited that the ability of *Artemia* to survive in wide concentration from sodium chloride. *Artemia* was examined against low sodium chloride levels of tap water in which larva was died after 24 hr. The best results of spawning and rearing of *Artemia* obtained at

concentration of 30-35%**0** artificial sea water.

It can concluded that improving the production of *Artemia* under controlled environmental conditions can be obtained with special care about the bacterial infection with adding a probiotic on food of high contains nutrients.

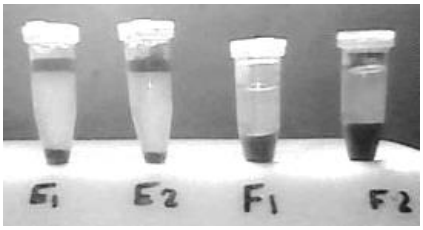

Fig (2): *Floating amount of used Artemia cysts in normal saline were appeared differ according to its type.*

Table (2): The reproductive characteristics

Strain	E1	E2	F1	F2
Start stage	larval	larval	larval	larval
First brood	oviparous	oviparous	oviparous	oviparous
percentage of oviparous up to two month	82%	84%	88%	85%
percentage of survival after two month	10%	12%	18%	14%

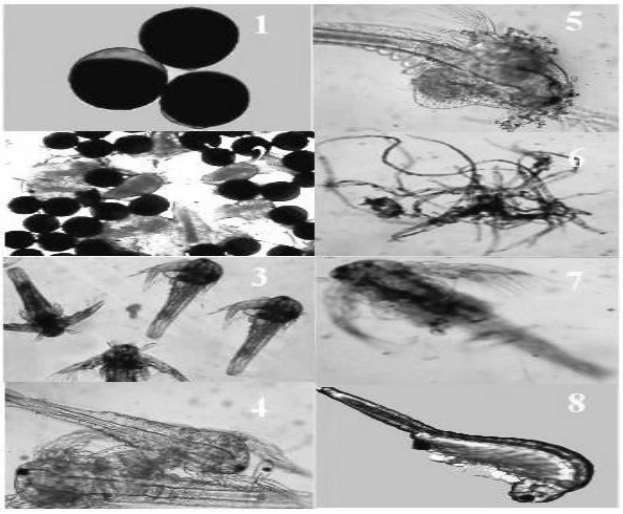

Fig. (3): *Direct microscopic examination of different growing stages of Artemia*

1- Cysts 2- Decapsulation and emerging of nauplii.

3, 4 and 5 growing stages. 6- Molting

7, 8 adult stage ovoviviparous.

Table (3): *Testing of feed types*

Days	**Rice Bran**	**Yeast**	**soy bean milk**
1	Instar 1	Instar 1	Instar 1
4			
7			
10			Instar 10
13	Instar 10	Instar 10	Pre-adult
16	Pre-adult	Pre-adult	Adult
19	Adult	Adult	Spawner
22		Spawner	
25	Spawner		

Table (4) *Biochemical characters of isolated organisms*

Character	***Isolate A***	***Isolate B***
AGS	KA	KA
Oxidase	+	+
Arginine dihydrolase	-	-
Gelatinase	+	+
Urease	-	-
Catalase	+	+
O/129(150 µg)	S	S
Ampicillin	R	R
Novobiocin	S	S
0% NaCl	-	-
3, 6 and 8% NaCl	+	+
10 and 12 % NaCl	+	-
Growth at 40°C	+	+
Growth at 42°C	+	-
Sucrose	+	-
D-Cellobiose	-	V
Lactose	-	-
Arabinose	-	+
Maltose and D-Mannitol	+	+
Gas from glucose	-	-
Indol	+	+
Methyl red	+	+
Voges-Proskauer	+	-
Suspected M. o. identified to:	*V.a*	*V.p*

Va: *Vibrio alginolyticus*, Vp: *Vibrio parahaemolyticus* TCBS: Thiosulfate-citrate-bile salts-sucrose. Modified cellobiose-polymyxin B-colistin; AGS: Arginine-glucose slant, S : Sensitive; V : Variable among strains; R : Resistant; KA : Slant alkaline (purple) / Butt acidic (yellow).

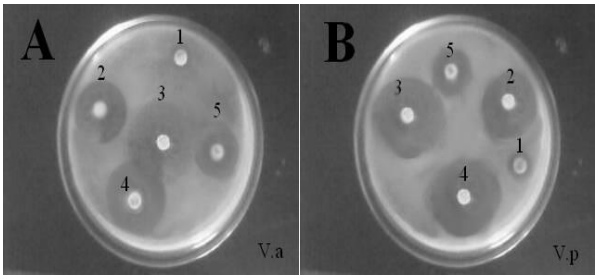

Fig. (4): *Inhibition zone of antimicrobial sensitivity test*

Table (5): *Antimicrobial sensitivity*

Antibiotic Discs name	Symbol & Conc.	Standards Zoon diameter interpretation			V.a		V.p	
		R	I	S	zone	result	zone	result
Ampicillin	AM 10 µ g	≤ 13	14-16	≥ 17	5	R	6	R
Chloramphenicol	C 30mcg	≤ 12	13-17	≥ 18	18	S	15	I
Cibrofloxacin	CIP 5mcg	≤ 15	16-20	≥ 21	22	S	21	S
Florfenicol	Ffc 30 µ g	≤ 14	15-18	≥ 19	22	S	19	S
Tetracycline	N 10 µ g	≤ 12	13-16	≥ 17	14	S	18	S

Table (6): Bacterial control techniques and survival rate

Methods	Techniques 1	Techniques 2	Techniques 3
Treatment	Natural (control)	Probiotics	Antibiotics
Survival %	6	14	11

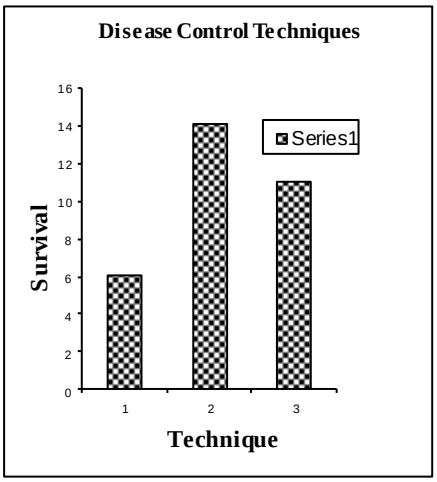

Fig. 5: *Techniques of diseases control*

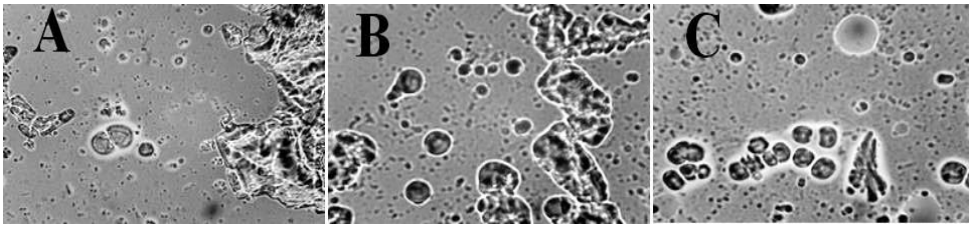

Fig. (6): Direct microscopic examination of sea water showed Cyanobacteria contamination

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الملخص العربى
دراسات حول المسببات البكتيرية والمشاكل التى تواجه التفريخ الإصطناعى للأرتيميا

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المعمل المركزى لبحوث الثروة السمكية بالعباسة– مركز البحوث الزراعية

تم اجراء الدراسه على 4 انواع من حويصلات الارتيميا اثنين منهم محلى المصدر مجمعة من البحيرات المرة ومريوط و اثنين من الانواع المستورده وذلك بهدف دراسة المشاكل المرضيه المصاحبه لعملية التفريخ و التربية. لهذا الغرض تم تصميم وحده رعاية مغلقه مكونه من 10 أحواض زجاجية يتم توزيع يرقات الارتيميا فيها بعد عملية التفريخ. تم اختبار ثلاثة انواع من المستحلبات الغذائيه (مستحلب القشرة الداخلية لحبة الارز, خميره الخبز, لبن الصويا) لمعرفة افضلهم لفترة الرعاية واوضحت النتائج أن لبن الصويا أفضل المعاملات التى وفرت الإحتياجات الغذائية للأرتيميا فى مراحل النمو المختلفة.

تم عمل العد البكتيرى لمياه البحر وعزل البكتريا منها حيث كان العدد للملى ليتر $2.6\text{-}2.8 \times 10^{7}$ وتم تصنيف نوعان من البكتريا من جنس الفيبريو كذلك تم عزل نفس البكتريا من اليرقات المرباه فى نفس المياه ولم يتم عزل بكتريا او فطريات من الحويصلات للانواع الاربعه.

تم إستخدام خميرة الخبز كنوع من البروبيتك وتتراسيكلين كمضاد حيوى وذلك فى محاولة للسيطرة على التواجد البكتيرى وكان إستخدام الخميرة هو الأفضل لهذا الغرض.

ومن المكن إختصار نتائج البحث فى إمكانية إستزراع الإرتيما بصورة إصطناعية فى دوائر مغلقة وفى ظروف بيئية محكمة مع إضافة بروبيوتك مثل خميرة الخبز إلى الغذاء وذلك لتوفير إحتياجات المفرخات منها.