Axenic culture of *Brachionus plicatilis* using antibiotics

Koushirou Suga[1], Yukari Tanaka[1], Yoshitaka Sakakura[1], Atsushi Hagiwara[2]

[1]Faculty of Fisheries, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, Japan

[2]Graduate School of Science and Technology, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, Japan

(*Author for correspondence: E-mail: sugakosi@nagasaki-u.ac.jp)

Key words: *Brachionus plicatilis*, antibiotic, bacteria free, resting egg disinfection.

This paper has not been submitted elsewhere in identical or similar form, not will it be during the first three months after its submission to Hydrobiologia.

Abstract

The rotifer *Brachionus plicatilis* culture is composed of complex microcosms including bacteria, protozoans, algae and fungus. Previous studies reported methods to establish axenic rotifer cultures, but further refinement of these techniques is needed, for molecular biological research which requires pure culture to isolate nucleic acids from rotifers only. In order to render rotifer culture axenic, we tested five antibiotics: ampicillin (Amp), chloramphenicol (Cp), kanamycin (Km), nalidixic acid (Na), and streptomycin (Sm) at 30-100 μg/ml. Except for Cp, which reduces rotifer reproduction, all other antibiotics at the tested concentrations did not affect rotifer reproduction or show any toxic effects. A rotifer disinfection method was finally established by treating the resting eggs with 0.25% (w/v) sodium hypochlorite (NaOCl) for 3 min, washing with sterilized sea water, and then exposing the neonates to an Amp, Km, Na, and Sm mixture. Using four nutrient media, we confirmed that this protocol renders the rotifer culture bacterial and fungus free. The axenic rotifer culture generated here is useful not only for genetic analysis of *Brachionus plicatilis*, but for studying the rotifer life cycle without bacterial influence.

Introduction

Brachionus plicatilis species complex is the essential live food for the initial stage of larval rearing of marine fishes (Hagiwara et al., 2001, 2007; Hagiwara, 2002), and is also studied in nutritional science for aquaculture (Scott, 1983; Hirayama et al., 1979; Kotani et al., 2009) and as a culture for probiotics (Douillet 2000a and 2000b; Hirata et al., 2004). It has also been widely used in ecotoxicological (Snell and Carmona, 1995; Snell and Janssen, 1995; Marcial et al., 2005), population dynamics (Hagiwara et al., 1994; Yoshinaga et al., 2003), and recent molecular biology studies (Suga et al., 2007a, 2007b and 2008; Denekamp et al., 2009; Oo et al., 2009). Rotifer culture forms a complex microcosm of bacteria, protozoa, algae, fungi and rotifers. Bacteria in rotifer culture affect various life history parameters of rotifer, such as growth and sexual reproduction (Hagiwara et al., 1994; Aoki and Hino, 1996). To eliminate interaction with microbes, several studies have been undertaken to obtain axenic rotifers which can be used in nutritional science, probiotics, molecular biology, and genetic studies of rotifers. Some of these studies reduced the bacterial load in the rotifer eggs, but complete disinfection of rotifer resting eggs has not been reported (Hagiwara et al., 1994; Balompapueng et al., 1997; Dhert et al., 1997; Douillet, 1998). Rombaut et al. (1999) reported that treating the resting eggs with glutaraldehyde renders the culture axenic. However, these researchers used only marine agar 2216 (ZoBell, 1941), which is a high-nutrient medium, for bacteria-free analysis. It is known that some bacteria from low-nutrient conditions do not grow in high-nutrient medium because of nutrient shock (Reasoner and Geldreich, 1985; Surman et al., 1994). Since rotifer culture is performed at low-nutrient conditions, analyzing bacteria in rotifer culture using only a high-nutrient medium such as marine agar 2216 is not optimal.

In this study, we established a method for axenic rotifer culture by treating rotifer resting eggs with sodium hypochlorite (NaOCl) and exposing the neonates to a mixture of antibiotics (ampicillin, kanamycin, nalidixic acid, and streptomycin). We confirmed that this protocol renders the rotifer culture bacteria- and fungus free, as analyzed by four different nutrient media (marine agar 2216, potato dextrose agar, R2A agar, and trypto-soy agar).

Materials and Methods

Materials

Brachionus plicatilis sensu stricto strain NH1L was used in this study. The strain originated from an outdoor eel culture pond in Mie Prefecture (Hagiwara et al., 1988), and was clonally cultured in Nagasaki University for more than 15 years. To obtain resting eggs, rotifers were cultured in diluted seawater at 22 ppt, at 25°C, in darkness, and fed *Nannochloropsis oculata*. The resting eggs produced were separated from the culture solution using a Pasteur pipette. The collected resting eggs were preserved at 4°C in darkness until use.

Treatment of resting eggs with NaOCl

Commercial-grade NaOCl was diluted with autoclaved sterilized Milli Q water to 0.25% (w/v) NaOCl. Resting eggs (about 2,000 eggs) stored in a 1.5 ml Eppendorf tube were washed with diluted NaOCl solution by shaking for 3 min. The eggs were poured on a sterilized plankton net (25 μm mesh), and subsequently washed by sterilized 22 ppt seawater to remove the NaOCl. The NaOCl-treated resting eggs were preserved at 4°C in darkness until use. All manipulations were carried out in a biological safety cabinet.

Effect on life history parameters of stem females treated with antibiotics

NaOCl-treated resting eggs were allowed to hatch in 10 ml of 22 ppt sterilized seawater at 25°C under continuous light. Hatched stem females were individually cultured in 96-well polystyrene cell culture plates (Iwaki) containing 200 μl of 22 ppt sterilized diluted seawater, fed axenic *Chlorella vulgaris* (3×10^6 cells/ml, Maruyama et al., 1989; Noda et al., 1996; Suga et al., 2007a) supplemented with 800 ng/ml vitamin V_{12} (which is essential for rotifer growth (Scott, 1983; Hirayama and Funamoto, 1983)) and antibiotics, including Amp, Cp, Km, Na, Sm and a 4 antibiotics mixture (Amp, Km, Na and Sm), for which each final concentration was 100, 40, 60, 30 and 60 μg/ml, respectively. The final concentrations of these antibiotics were determined based on the concentrations generally used for analysis of the bacterial antibiotic resistance (Raleigh et al., 2002). Each antibiotic or ethanol (EtOH, solvent for Cp, final concentration was 0.07% (v/v)), was tested 10 individually cultured neonates from resting eggs. After the addition of rotifers, the plates were incubated at 25°C in darkness. The plates were inspected every 12h, and the spawning and hatched eggs were counted and the neonates removed. Stem females were transferred to new wells and given freshly prepared food and antibiotics daily until death. The lifespan, fecundity and hatching rate of amictic eggs were

determined. The experiment was replicated three times.

Effect on life history parameters of F_1 and F_2 amictic females

Six amictic females (F_1) selected from stem females which were treated with Cp or Mix, or untreated with antibiotic, were hatched from NaOCl-treated resting eggs. These F_1 were individually cultured in 96-well plates in the same condition as described above, but without antibiotics. The plates were inspected every 12h to determine the lifespan, and to count the spawning and hatched eggs using a stereomicroscope. Each amictic female (F_1) was transferred to a new well with freshly prepared seawater containing axenic *Chlorella*. Six offspring (F_2), which were obtained from the F_1, were individually cultured to determine their life history parameters using the same method as in F_1.

Microbial analysis by agar plating method

To determine the presence of bacteria in the rotifer culture medium, 30 stem females, which were obtained by hatching resting eggs treated with NaOCl, were cultured in screw glass vials containing 5 ml of sterilized 22 ppt seawater with axenic *Chlorella* (3×10^6 cells/ml) containing vitamin V_{12} and antibiotics. The final concentration of each antibiotic was the same as in the individual culture condition. After 8 days of cultivation, each culture solution with or without dilution from 10 to 10,000 times using sterilized 22 ppt seawater were plated into marine agar 2216 (Difco), potato dextrose agar (Eiken), R2A agar (Difco) and trypto-soy agar (Eiken). The plates were incubated from 2 days to 2 weeks at 25^{o}C, and the colony forming unit (CFU/ml) was calculated from the number of colony on each plate.

Statistical analysis

One-way analysis of variance (ANOVA) was conducted to identify significant differences among the groups in number of offspring, hatching rate of amictic eggs and lifespan with or without antibiotics treatment. In the cases in which significant differences were detected by ANOVA ($p<0.05$), multiple comparisons were conducted using the Tukey-Kramer test to identify which groups were significantly different.

Results

Effect on life history parameters of stem females treated with antibiotics

All antibiotics at the tested concentrations had no effect on the lifespan of stem females (Table 1). The results of treatment with Amp, Km, Na, Sm or their mixture showed that the lifespan, fecundity, and hatching rate of amictic eggs were not significantly different from the control (untreated antibiotics). In contrast, the fecundity and hatching rates of amictic eggs were significantly decreased in Cp treated stem females. The life history parameters of stem females treated with 0.07% (v/v) EtOH, used as a solvent of Cp, were not different from the control. These results showed that Cp had a toxic effect on germ cell development and development of eggs but not on the lifespan of stem females.

Effect on life history parameters of F_1 and F_2 amictic females

The fecundity of F_1 derived from stem females treated with antibiotics mixture or Cp was significantly lower than the control (Table 2). The lifespan of F_1 from stem females treated only with Cp was significantly decreased. Cp had no toxic effect on the hatching rate of amictic eggs spawned by F_1, but all F_2 from stem females treated with Cp had innate malformation and these abnormal neonates were sterile (data not shown). In contrast, the lifespan, fecundity and hatching rates of amictic eggs were not significantly different from the F_2 generation on control.

Microbial analysis

The bacterial colonies of rotifer culture medium of stem females not treated with NaOCl and antibiotics were about 10^5 CFU/ml on marine agar 2216, R2A agar and trypto-soy agar and 10^3 CFU/ml on potato dextrose agar. Several colonies were detected on potato dextrose agar plates, but none on marine agar 2216, R2A agar and trypto-soy agar plates, from the rotifer culture medium hatched from resting eggs treated with NaOCl only. At all agar media tested, no bacterial colony was detected on rotifer culture medium, which was cultured from antibiotics treatment of stem females derived from NaOCl-treated resting eggs. These results showed that antibiotics treatment of stem females followed by washing of the resting eggs with NaOCl was effective for creating a bacteria-free rotifer culture.

Discussion

Rotifer batch culture is a complex microcosm of bacteria, protozoa, fungi and rotifers (Miyakawa and Muroga, 1988). Several authors have reported methods for obtaining axenic culture of rotifer by treating the resting eggs with disinfecting compounds such as NaOCl, glutaraldehyde, and antibiotics used independently or in combination (Dougherty et al., 1961; Hirayama et al., 1979; Hagiwara et al., 1988; Douillet 1998; Rombaut et al., 1999). NaOCl is one of the most useful and important sterilizers, but has very low sterilizing power for *Bacillus* spores (Kitaori and Takahashi, 2005). Antibiotics do not have the immediate disinfecting ability of compounds such as NaOCl, and are also not effective for sterilizing spores and eukaryotes, including fungus. In some studies, antibiotics were used to wash rotifer resting eggs, and were found to remove bacteria completely (Dougherty et al., 1961; Hirayama et al., 1979). In this study, we examined the effect of antibiotics on the rotifer, and established a method to produce axenic rotifer culture by treating rotifers with antibiotics for several days followed by a NaOCl wash of the resting eggs.

Douillet (1998) reported that the highest hatching rate for NaOCl treatment resulting in bacteria-free rotifers was obtained after 3 min exposure to 0.5% (w/v) NaOCl solution. We applied the same method to resting eggs, but the eggs burst, resulting in death. Based on the procedure of Douillet, NaOCl was removed by suction. In this study we used a plankton net to collect the resting eggs after NaOCl treatment, and then washed the eggs with sterilized 22 ppt seawater by gentle pipetting. However, NaOCl could not be easily removed using this method, and, as mentioned above, the resting eggs burst when using 0.5% (w/v) NaOCl solution. Therefore, we used a lower concentration (0.25% (w/v) NaOCl for 3 min).

Antibiotics are categorized as bacteriocidal and bacteriostatic, and their mechanism of action includes inhibition of bacterial cell wall synthesis, protein synthesis, DNA synthesis and translocation of peptidyl tRNA (Raleigh et al., 2002). In order to kill various kinds of bacteria, it is necessary to use a broad antimicrobial spectrum of various kinds of antibiotics which have a different mechanism of action. In this study, we selected three bacteriocidal type antibiotics, Amp, Km and Sm, and two bacteriostatic type antibiotics, Cp and Na (Raleigh et al., 2002). Our experiment showed that Amp, Km, Na, Sm or their mixture had no significant effect on the life history parameters of the rotifer at the concentrations used, but Cp was toxic to the reproduction and hatchability of rotifers. Cp is known to inhibit protein synthesis by interacting with the 50S ribosomal subunit and inhibiting the peptidyl transferase reaction (Raleigh et al., 2002). Amikura et al. (2005) reported that Cp impairs

production of germ cell-less protein and disrupts pole cell formation in *Drosophila* embryos. We suggest that Cp affects rotifer germ cells and embryos in the same manner, resulting in a decrease of the fecundity and hatching rate of amictic eggs which persists in successive generations (Table 2). The fecundity decreased with the F_1 generation from stem females treated with the antibiotics mixture. The decrease was much larger in the F_1 generation from stem females treated with Cp. All F_2 from stem females treated with Cp showed innate malformation and these abnormal neonates had no ability to spawn.

These F_2 females were not directly treated with antibiotics. Instead, F_1 were exposed to antibiotics during the embryogenesis stage, while inside the stem females. The stem females were treated with Cp from the neonate to adult stages, but not during the embryogenesis stage. The antibiotics had no effect on the life history parameters of stem females. These results suggest that the mixture of four kinds of antibiotics weakly inhibited only early germ cell development in the embryogenesis stage, and that Cp has the potency to strongly inhibit embryonic development and early germ cell development in the embryogenesis stage. The life history parameters of F_2 were not significantly different from the control, so that the effects of antibiotics are not multigenerational. These results suggest that Cp is useful for creating permanent transgenic rotifer using the chloramphenicol acetyltransferase gene, which is a Cp inactivator, as a marker.

In order to check the disinfecting capacity of antibiotics in rotifer culture after disinfectant treatment, several authors have used high-nutrient agar such as marine agar 2216. A single rotifer may harbor as many as 10^8 bacterial CFU and a culture typically contains 10^3-10^6 bacterial CFU/ml (Miyakawa and Muroga, 1988). To detect the various kinds of bacteria present in the rotifer culture, we used 4 kinds of agar media: marine agar 2216 for detecting marine bacteria, potato dextrose agar and trypto-soy agar for bacteria and fungus, and R2A agar for bacteria. It is known that high-nutrient culture medium is not appropriate for culturing bacteria from low-nutrient conditions such as natural water (Surman et al., 1994; Bartscht et al., 1999; Reasoner, 2004). R2A is a low-nutrient culture medium, and nutrient shock is avoided by culturing the bacteria from it. We detected bacterial colonies ranging from 10^3 to 10^5 in 1 ml rotifer culture from non treated resting eggs. The bacterial colonies were not detected on marine agar 2216, R2A agar and trypto-soy agar plates, but several colonies were detected on potato dextrose agar plate in 1 ml rotifer culture from NaOCl-treated resting eggs. At all agar plates, no bacterial colony was detected on rotifer culture from antibiotics treatment

of stem females derived from NaOCl-treated resting eggs. These results show that the rotifer culture from NaOCl-treated resting eggs is not completely bacteria free, and that high-nutrient agar, such as marine agar 2216 is insufficient for detecting bacteria from rotifer culture. We could not detect any bacterial colony on the four kinds of agar plates after 1 month cultivation of this culture solution (data not shown). Using the same rotifers, we previously constructed a cDNA library and did not find any sequenced clone related to bacteria and fungi (Suga et al., 2007a). Taken together, these results suggested that the rotifer culture using this method is completely axenic.

In summary, we found that the antibiotics mixture (Amp, Km, Na and Sm) had no multigenerational effect on rotifer, while Cp caused critical damage to embryonic development and early germ cell development. Using the combination of NaOCl to wash the resting eggs and antibiotics mixture (Amp, Km, Na and Sm) treatment of amictic females for 8 days, rotifers could be cultured in a completely bacteria-free condition. The use of several types of culture media, such as high and low-nutrient media, was effective in detecting all bacterial colonies in the rotifer culture.

Acknowledgments

A part of this research was supported by a Grant in Aid for Scientific Research (B), 2009-2011, No. 21380125, from the Ministry of Education, Culture, Sports, Science and Technology of Japan to A. H. We would like to thank Dr. Helen S. Marcial for her review and comments.

References

Aoki, S. & A. Hino, 1996. Nitrogen Flow in a Chemostat Culture of the Rotifer *Brachionus plicatilis*. Fisheries Science 62: 8-14.

Amikura, R., K. Sato & S. Kobayashi, 2005. Role of mitochondrial ribosome-dependent translation in germline formation in *Drosophila* embryos. Mechanisms of Development 122: 1087-1093.

Balompapueng, M. D., N. Munuswamy, A. Hagiwara & K. Hirayama, 1997. Effect of disinfectants on the hatching of marine rotifer resting eggs *Brachionus plicatilis* Müller. Aquaculture Research 28: 559-565.

Bartscht, K., H. Cypionka & J. Overmann, 1999. Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. FEMS Microbiology Ecology 28: 249-259.

Denekamp, N. Y., M. A. S. Thorne, M. S. Clark, M. Kube, R. Reinhardt & E. Lubzens, 2009. Discovering genes associated with dormancy in the monogonont rotifer *Brachionus plicatilis*. BMC Genomics 10: 108.

Dhert, Ph., K. Schoeters, P. Vermeulen, J. Sun, S. Gao, Z. Shang, X. Naihong, H. Van Duffel & P. Sorgeloos, 1997. Production, disinfection and evaluation for aquaculture applications of rotifer resting eggs from Bohai Bay, P.R. of China. Aquaculture International 5: 105-112.

Dougherty, E. C., B. Solberg & D. J. Ferral, 1961. The first axenic cultivation of a rotifer species. Experientia 17:131-132.

Douillet, P., 1998. Disinfection of rotifer cysts leading to bacteria-free populations. Journal of Experimental Marine Biology and Ecology 224: 183-192.

Douillet, P. A., 2000a. Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions 1. Evaluation of commercial products and pure isolates. Aquaculture 182: 249-260.

Douillet, P. A., 2000b. Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions 2. Use of single and multiple bacterial probiotics. Aquaculture 182: 241-248.

Hagiwara, A., A. Hino & R. Hirano, 1988. Comparison of resting egg formation among five Japanese stocks of the rotifer *Brachionus plicatilis*. Nippon Suisan Gakkaishi 54: 577-580.

Hagiwara, A., K. Hamada, S. Hori & K. Hirayama, 1994. Increased sexual reproduction in *Brachionus plicatilis* (Rotifera) with the addition of bacteria and rotifer extracts. Journal of Experimental Marine Biology and Ecology 181: 1-8.

Hagiwara, A., W. G. Gallardo, M. Assavaaree , T. Kotani & A. B. de Araujo, 2001. Live food production in Japan: recent progress and future aspects. Aquaculture 200: 111-127.

Hagiwara, A., 2002. Frist live food of marine fish: biological function of rotifers and application to larval rearing. Suisan Zoshoku 50: 473-478. (In Japanese)

Hagiwara, A., K. Suga, A. Akazawa, T. Kotani, & Y. Sakakura, 2007. Development of rotifer strains with useful traits for rearing fish larvae. Aquaculture 268: 44-52.

Hirata, H., O. Murata, S. Yamada, H. Ishitani & M. Wachi, 2004. Probiotic culture of the rotifer *Brachionus plicatilis*. Hydrobiologia 387/388: 495-498.

Hirayama, K., K. Takagi & H. Kimura, 1979. Nutritional effect of eight species of marine phytoplankton on population growth of the rotifer, *Brachionus plicatilis*. Nippon Suisan Gakkaishi 45: 11-16.

Hirayama, K. & H. Funamoto, 1983. Supplementary effect of several nutrients on nutritive deficiency of baker's yeast for population growth of the rotifer *Brachionus plicatilis*. Bulletin of the Japanese Society of Scientific Fisheries 49: 505-510.

Kitaori, N. & M. Takahashi, 2005. Improvements in the sterilization performance of sodium hypochlorite. Electrochemistry 73: 141-144.

Kotani, T., T. Genka, H. Fushimi, M. Hayashi, K. Dierckens & P. Sorgeloos, 2009. Effect of cultivation methods on nutritional enrichment of euryhaline rotifer *Brachionus plicatilis*. Fisheries Science 75: 1-10.

Noda, K., N. Ohno, K. Tanaka, N. Kamiya, M. Okuda, T. Yadomae, K, Nomot & Y. Shoyama, 1996. A water-soluble antitumor glycoprotein from *Chlorella vulgaris*. Planta Medica 62: 423-426.

Marcial, H. S., A. Hagiwara & T. W. Snell, 2005. Effect of some pesticides on reproduction of rotifer *Brachionus plicatili*s Müller. Hydrobiologia 546: 569-575.

Maruyama, I., Y. Ando, T. Maeda & K. Hirayama, 1989. Uptake of vitamin B12 by various strains of unicellular algae *Chlorella*. Nippon Suisan Gakkaishi 55: 1785-1790.

Miyakawa, M. & K. Muroga, 1988. Bacterial flora of cultured rotifer *Brachionus plicatilis*. Suisan Zoshoku 35: 237-243.

Oo, A. K., G. Kaneko, M. Hirayama, S. Kinoshita & S. Watabe, 2009. Identification of genes differentially expressed by calorie restriction in the rotifer (*Brachionus plicatilis*). Journal of Comparative Physiology. B: Biochemical, Systemic, and Environmental Physiology. DOI:10.1007/s00360-009-0389-6

Raleigh, E. A., K. Elbing & R. Brent, 2002. Selected topics from classical bacterial genetics. In Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Sediman, J. A. Smith & K. Struhl (eds), Current Protocol In Molecular Biology. Wiley, New York, NY: Chapter 1, Unit 1.4.

Reasoner, D. J., 2004. Heterotrophic plate count methodology in the United States. International Journal of Food Microbiology 92: 307-315.

Reasoner, D. J. and Geldreich, E. E. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49: 1-7.

Rombaut, G., Ph. Dhert, J. Vandenberghe, L. Verschuere, P. Sorgeloos & W. Verstraete, 1999. Selection of bacteria enhancing the growth rate of axenically hatched rotifers (*Brachionus plicatilis*). Aquaculture 176: 195-207.

Scott, J. M., 1983. Rotifer nutrition using supplemented monoxenic cultures. Hydrobiologia 104: 155-166.

Snell, T. W. & M. J. Carmona, 1995. Comparative toxicant sensitivity of sexual and asexual reproduction in the rotifer *Brachionus calyciflorus*. Environmental Toxicology and Chemistry 14: 415-420.

Snell, T. W. & C. R. Janssen, 1995. Rotifers in ecotoxicology: a review. Hydrobiologia 313/314: 231-247.

Suga, K., D. B. Mark Welch, Y. Tanaka, Y. Sakakura & A. Hagiwara, 2007a. Analysis of expressed sequence tags of the cyclically parthenogenetic rotifer *Brachionus plicatilis*. PLoS ONE 2: e671.

Suga, K., Y. Tanaka, Y. Sakakura & A. Hagiwara, 2007b. Inheritance of mitochondrial DNA in the rotifer *Brachionus plicatilis*. Hydrobiologia 593: 167-173.

Suga, K., D. B. Mark Welch, Y. Tanaka, Y. Sakakura & A. Hagiwara, 2008. Two circular chromosomes of unequal copy number make up the mitochondrial genome of the rotifer *Brachionus plicatilis*. Molecular Biology and Evolution 25: 1129-1137.

Surman, S. B., L. H. G. Morton & C. W. Keevil, 1994. The dependence of *Legionella pneumophila* on other aquatic bacteria for survival on R2A medium. International Biodeterioration and Biodegradation 33: 223-236.

Yoshinaga, T., G. Kaneko, S. Kinoshita, K. Tsukamoto & S. Watabe, 2003. The molecular mechanisms of life history alterations in a rotifer: a novel approach in population dynamics. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 136: 715-722.

ZoBell, C. E., 1941. Studies on marine bacteria. I. The cultural requirement of heterotrophic aerobes. J. Marine Research 4: 42-75.

Table 1 Effect on life history parameters of stem females treated with antibiotics.

NaOCl treatment for resting eggs	Antibiotics treatment for stem females	Lifespan (days)	Fecundity (eggs/female)	Hatching (%) of amictic egg
-	-	10.6±0.5	10.7±0.8	99.2±1.4
+	-	12.7±2.1	11.4±1.9	98.9±0.3
+	Amp	13.4±0.6	8.4±1.5	99.0±1.7
+	Cp	12.7±1.4	4.0±1.1*	29.9±9.3*
+	Km	13.9±1.8	8.5±1.5	100.0±0.0
+	Na	12.2±2.9	8.7±1.2	97.4±2.9
+	Sm	14.6±1.3	9.1±1.4	100.0±0.0
+	Mix	14.5±0.7	9.3±1.4	99.3±0.7
+	EtOH	12.5±0.8	10.4±2.5	99.7±0.5

Data indicate means and standard deviation (n = 3). +, treated; -, untreated; *, Tukey-Kramer test, $p < 0.05$

Table 2 Effect on life history parameters of F_1 and F_2 amictic females

Antibiotics treatment	Lifespan (days)		Fecundity (eggs/female)		Hatching (%) of amictic egg	
for stem females	F_1	F_2	F_1	F_2	F_1	F_2
-	14.2±2.5^b	11.8±2.3	13.0±1.3^c	11.7±5.0	98.8±2.9	98.7±3.1
Mix	14.3±1.8^b	13.2±4.1	9.3±1.4^b	13.2±1.7	97.9±5.1	96.8±5.3
Cp	5.8±2.1^a	ND	0.7±1.2^a	ND	100.0±0.0	ND

The resting eggs were treated with NaOCl. Data indicate means and standard deviation (n = 6). -, untreated; ND, All F_2 from stem females treated with Cp showed innate malformation and these abnormal neonates had no ability to spawn; a<b<c, Tukey-Kramer test, $p < 0.05$.

Table 3 Microbial analysis by agar plating method after 8 day culture at 25 °C.

NaOCl treatment for resting eggs	Antibiotics treatment for females	Medium CFU/ml			
		MA	PDA	R2A	TSA
-	-	5.7 x 10^5	5.0 x 10^3	5.9 x 10^5	2.3 x 10^5
+	-	0	26.7	0	0
+	Amp	0	0	0	0
+	Cp	0	0	0	0
+	Km	0	0	0	0
+	Na	0	0	0	0
+	Sm	0	0	0	0
+	Mix	0	0	0	0

+, treated; -, untreated. MA, marine agar 2216; PDA, potato dextrose agar; R2A, R2A agar (Reasoner and Geldrich, 1985); TSA, trypto-soy agar.