Effect of different concentration of profitable Bacillus on bioremediation of common carp (Cyprinus carpio) pond discharge

M Naderi Samani*1, H Jafaryan2, H Gholipour2, M Harsij2, M Farhangi 2

1Ph.D student of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
2Department of Fishery, Gonbad University of Agriculture Sciences and Natural Resources, Gonbad, Iran

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Abstract

A 4 days study was conducted to determine the effects of different concentration of bioaugmentor bacterial strain Bacillus licheniformis, B. subtilis, B. polymyxa, B. laterosporus and B. circulans (Protexin Aquatech, UK) on adjustment of parameters of water quality as TAN, NO2-N, NO3-N and turbidity from the effluent of Common Carp ponds. Effects of time and concentration were studied as a completely randomized split-plot design. Time and concentration and their interaction had a significant difference (P<0.05) on changes of total ammonium nitrogen, nitrate nitrogen, nitrite nitrogen and turbidity. The total counts of bacteria recorded in the water of bioremediated tanks were also lower than that in the control tank.

Keywords: Bacillus, bacterial treatment, parameters of water quality, bioremediation

Introduction

During the past twenty years, aquaculture and mariculture have significantly improved. However, the effluent of aquaculture activity without treatment procedure increases pollution of environmental acceptor water and damages the ecology of culture areas. This is a result of aquaculture wastewater study, which contains significant amounts of nutrients such as nitrogen, phosphorus and the amount of organic material reduces the water quality and can be a potential condition for pathogenic microorganisms quickly grow and distribute (Zhou, L.i, Jun & Bo 2009). Efficient use and reuse of water resources requires affordable and efficient strategies which help to reduce pollution of wastewater. Chemical and biological treatment process are two main
methods during the treatment of wastewater. Chemical treatment are more effective for the removal of non-biodegradable materials than the biological methods but the disadvantages of chemical treatment are massive (Makridis, Bergh, Skjermo & Vadstein 2001; Jafaryan, Soltani, Noferesti & Ebrahimi 2011), thus biological treatment of wastewater is Supported in the last few decades (Akpor & Muchie 2010).

In recent decades bioremediation by probiotics has become highly regarded and many researchers around the world are engaged in research and study associated with the influence of useful bacteria on treatment contaminated water and reduction of pollutants of water source. In recent years, beneficial results and experiences have been obtained from these studies (Boyd, Hollerman, Plumb & Saeed 1984). Bioremediation is a term that is used to improve water quality in aquaculture by performance of probiotics. Treatment with the help of probiotics as a biological control method is considered. biocntrol is related to the elimination of waste like parasites or specific pathogens (Moriarty 1998). Moriarty (1998) proposed the microbial probiotics as water additives. Burford, Thompson, McIntosh, Bauman & Pearson (2003); Devaraja, Yusoff & Shariff (2002); Queiroz & Boyd (1998); Vezzulli, Puzzo & Fabiano (2004) reported that the results of bioremediation experiments showed that elimination of organic pollutants can be done with microbial products and promote water quality.

According to the published findings, application of beneficial bacteria in the water of fish pond is based on two principles: biocontrol with the goal of being antagonistic to pathogens (Gatesoupe 1999; Moriarty 1998; Nogami & Maeda 1992; Skjermo & Vadstein 1999; Rengpipat, Phianphak, Piyatiratitivorakul & Menasveta 1998). Especially bacteria found in companies, as biocontrol agents appears worthwhile instead of the negative effects of antibiotics (Abraham, Shanmugham, Uma, Palaniappan & Dhevendran 2001).

Bioremediation for controlling water quality (van Rijn, Fonarev & Berkowitz 1995; van Rijn & Nussinovitch 1997; Prabhu, Nazar, Rajagopal & Khan 1999; Queiroz & Boyd 1998) that Singh & Radhika (2001) said. Most probiotics used in aquaculture as biological control agents belong to the Lactic Acid Bacteria (Lactobacillus, Carnobacterium etc.), Vibrio (Vibrio alginolyticus), Bacillus, and Pseudomonas.

The main sources of nitrogenous substances in rearing fish culture are fish excretion and the matter that is released from the sediments during the conversion of organic matter into inorganic substances, although nitrogen fixation by cyanobacteria and atmospheric diffusion are important (Ayyappan & Mishra 2003). Total ammonia nitrogen (TAN = NH3-N + NH4-N) removal in a recirculating aquaculture is very important because of NH3-N.
is toxic even at low concentrations (Eding, Kamstra, Verreth, Huisman & Klapwijk 2006). Nitrogenous substances contained in sewage discharges have the important role in eutrophication of receiving waters and one of the most economical processes for nitrogen removal from sewage is a biological nitrification–denitrification (Gupta & Gupta 2001).

Mevel and Prieur (Mevel & Prieur 2000) reported that Bacillus strains are known to be involved in heterotrophic nitrification. Bacillus sp. is one of the popular microbial products for aquaculture, in the Chinese market Liu & Han (2004) stated the popularity of Bacillus sp is due to its established manufactory technique, mass supply, simple preservation and convenient transportation. The role of Bacillus sp. due to its adaptation in aquatic environments is very important. Bacillus is a genus of Gram-positive, rod-shaped bacteria and a member of the phylum Firmicutes, spore forming bacteria, used generally as a probiotic due to its high resistance against extreme environmental conditions and also its high reproducibility and low cost of increased production is considered a beneficial microorganism than others (Wang, Li & Lin 2008).

Moriarty (1998) stated that controlled species of vibrio luminous, by adding many strains of Bacillus in the shrimp ponds (Penaeidae), finally increased shrimp survival due to a direct effect of Bacillus performance on animal health and decomposing organic matter and promote water quality. In two different investigations, Jafaryan et al (2011) found that adding probiotics Bacillus in rearig water of Cyprinus carpio and Ctenopharyngodon idella larvae enhanced the growth of the fish.

Ghosh, Sinha & Sahu (2008) stated that the use of Bacillus subtilis in the rearing water of the livebearing fishes resulted in improvement of water quality. Use of Bacillus subtilis in the rearing water of the livebearing fishes resulted in improvement of water quality organic matter and nitrogen removal from reclaimed wastewater used as landscape water was carried out by Zhao, Hu, Chen, Zha & Liang (2009) by using the Bacillus cereus.

This study was designed to investigate the effects of different concentrations of probiotics at different times on bioremediation. This article tries to find the best time and concentration of probiotics on adjustment of water quality parameters in rearing tanks as the living environment of reared fish, bioremediation of effluent, re-use of water resources, to prevent pollution of water acceptor and to help preserve valuable natural environmental organisms.

**Material and Method**

**Experimental Materials**

The probiotic Bacillus was prepared from Protexin Co (Iran-Nikotak). The blends of
probiotic bacilli (*Bacillus licheniformisi, B. subtilis, B. polymyxa, B. laterosporus* and *B. circulans*) from suspension were provided. Three concentrations of bacterial suspension, $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$ CFU/L was determined by optical density at 610 nm in a spectrophotometer and the colony forming unit (CFU) of probiotic bacillii were tested by microbial culture in Tryptic Soy Agar (TSA). The effluent prepared from cultivation system of Common Carp (*Cyprinus carpio*) in fishery lab of University of Gonbade kavos, Iran.

**Experimental Setup**

This experiment was conducted in 4 treatments, each with three replicates. 12 plastic tanks with a volume of 3 liters of wastewater was prepared. Treatment 2, 3 and 4 incubated by the blend of bacilli were added directly to at a concentration of $1 \times 10^6$, $1 \times 10^7$ and $1 \times 10^8$ CFU/ liter, respectively and in control treatment (T-1) didn’t add any bacilli. All of trial tanks were aerated water temperature was 21-23 °C.

**Measured parameters**

At the starting of the experiment and after inoculating of bacteria into the waste water, the changes of water quality parameters such as TAN, NO$_2$-N, NO$_3$-N and turbidity was measured at intervals of 24, 48 and 72 h. NO$_2$-N, NO$_3$-N, TAN by spectrophotometer Manufacturing CoHANNA (HI83200 Model). turbidity with a portable multi-line to be measured after calibration.

**Total count of bacteria in water**

Every day, water samples were collected in sterile containers, serially diluted and the total bacterial counts were determined by spread plating in triplicate on TSA with incubation at 30°C for 48 h (Ghosh *et al.* 2008).

**Data Analysis**

Analyzed in a completely randomized split-plot design using MSTATC statistical software to compare LSD procedure was performed at the 5% level. Before analysis, data normality was tested. he results of the total counts of bacteria were processed by log10 transformation.

**Result**

The parameters of water quality are presented in Table 1. The results show the time and concentrations and their interaction effect on NO$_2$-N, NO$_3$-N, TAN turbidity and total count of bacteria. The different concentrations (0, $10^6$, $10^7$, $10^8$ cfu/l) are indicated by 1,2,3,4 and different times (0, 24, 48, 72) are introduced by 1, 2, 3, 4.

Significant difference was observed for these parameters between the treatment groups. The highest TAN, lowest NO$_3$-N was obtained in control treatment T1at the first day and the lower TAN, highest NO$_3$-N was observed in experimental treatment T4 after 72 h (Table2).

The highest turbidity was obtained in control treatment T1at the first day and after 72 h was not significantly difference between 2, 3 and 4 treatment.
Table 1 Variance analysis: the effect of time, concentration and their interaction on NO$_2$-N, NO$_3$-N, TAN and turbidity

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>NO$_2$-N</th>
<th>NO$_3$-N</th>
<th>TAN</th>
<th>Turbidity</th>
<th>Total count colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>time</td>
<td>3</td>
<td>1.52**</td>
<td>4.38**</td>
<td>7.042**</td>
<td>6.24**</td>
<td>5.14**</td>
</tr>
<tr>
<td>r × time</td>
<td>8</td>
<td>0.009</td>
<td>0.16</td>
<td>0.01</td>
<td>0.047</td>
<td>0.23</td>
</tr>
<tr>
<td>Concentration</td>
<td>3</td>
<td>0.04**</td>
<td>1.24**</td>
<td>0.66**</td>
<td>1.37**</td>
<td>0.67**</td>
</tr>
<tr>
<td>Time × Concentration</td>
<td>9</td>
<td>0.201**</td>
<td>1.62**</td>
<td>0.01**</td>
<td>0.21**</td>
<td>0.2**</td>
</tr>
<tr>
<td>r × Time × Concentration</td>
<td>24</td>
<td>0.006</td>
<td>0.2</td>
<td>0.007</td>
<td>0.034</td>
<td>0.076</td>
</tr>
<tr>
<td>Coefficient of Variance</td>
<td>11</td>
<td>11.34</td>
<td>8.45</td>
<td>4.56</td>
<td>3.38</td>
<td>4.07</td>
</tr>
</tbody>
</table>

Significant difference at level 0.01 indicated by **

Table 2 Changes in the water quality parameters. The different concentrations (0, 10$^6$, 10$^7$, 10$^8$ cfu/l) are indicated by 1, 2, 3, 4 and different times (0, 24, 48, 72) are introduced by 1, 2, 3, 4.

<table>
<thead>
<tr>
<th>time</th>
<th>Conc.</th>
<th>NO$_2$-N (mg/l)</th>
<th>NO$_3$-N (mg/l)</th>
<th>TAN (mg/l)</th>
<th>Turbidity (NTU)</th>
<th>Total count CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.28±0.006f</td>
<td>5.26±0.25de</td>
<td>2.58±0.047a</td>
<td>6.4±0.14a</td>
<td>7.53±0.41a</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.28±0.006f</td>
<td>5.26±0.25de</td>
<td>2.58±0.047a</td>
<td>6.4±0.14a</td>
<td>7.53±0.41a</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>0.28±0.006f</td>
<td>5.26±0.25de</td>
<td>2.58±0.047a</td>
<td>6.4±0.14a</td>
<td>7.53±0.41a</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0.28±0.006f</td>
<td>5.26±0.25de</td>
<td>2.58±0.047a</td>
<td>6.4±0.14a</td>
<td>7.53±0.41a</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.77±0.14c</td>
<td>4.9±0.26ef</td>
<td>2.42±0.07ab</td>
<td>5.9±0.3b</td>
<td>6.89±0.25b</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.58±0.06de</td>
<td>4.36±0.51f</td>
<td>2.07±0.15cde</td>
<td>4.9±0.18e</td>
<td>6.54±0.6bcd</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.52±0.042e</td>
<td>4.36±0.20f</td>
<td>1.73±0.15bcd</td>
<td>5.2±0.20cd</td>
<td>6.37±0.32cd</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.49±0.097e</td>
<td>4.73±0.40ef</td>
<td>1.99±0.11bc</td>
<td>4.6±0.05ef</td>
<td>6.65±0.37bcd</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0.73±0.046c</td>
<td>5.36±0.20cde</td>
<td>2.06±0.09def</td>
<td>6.3±0.15a</td>
<td>7.54±0.31a</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.71±0.046cd</td>
<td>6.06±0.51bc</td>
<td>1.31±0.1defg</td>
<td>5.5±0.15c</td>
<td>6.91±0.13b</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.55±0.034e</td>
<td>5.83±0.20cd</td>
<td>1.23±0.07efgh</td>
<td>5.2±0.33cd</td>
<td>6.79±0.13bc</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0.74±0.063c</td>
<td>5.43±0.15cde</td>
<td>1.16±0.07def</td>
<td>5.3±0.11c</td>
<td>6.71±0.31bcd</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1.12±0.030b</td>
<td>4.43±0.15f</td>
<td>1.40±0.04fgh</td>
<td>5.2±0.52cd</td>
<td>6.32±0.28d</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1.29±0.17a</td>
<td>5.3±1.1de</td>
<td>0.86±0.06fgh</td>
<td>4.4±0.76f</td>
<td>6.45±0.16bcd</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1.05±0.16b</td>
<td>6.8±0.50ab</td>
<td>0.9±0.1h</td>
<td>4.5±0.71f</td>
<td>5.48±0.22e</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1.11±0.063b</td>
<td>7.3±0.36a</td>
<td>0.7±0.04g</td>
<td>4.5±0.78f</td>
<td>5.63±0.28e</td>
</tr>
</tbody>
</table>

Values are means ± SD of treatment groups. Values in the same column with different superscripts for each species are significantly different (P<0.05).

Discussion

In the natural aquatic environment, energy and carbon source were provided for survival and growth of Microorganisms using the contaminant, thus they play important roles in the geological cycle of elements and
transformation of natural chemicals (Watanabe 2002).

The result showed that administration of the *Bacillus sp.* via direct inoculation to effluent rearing tanks the result showed that administration of the *Bacillus sp.* via direct inoculation to effluent rearing tanks significantly decreased total ammonia nitrogen (TAN = NH₃-N + NH₄-N) in bacterial treatment compared with blank treatment but NO₃-N significantly increased in experimental treatment. Koops and Moller (1992) presented Bacilli contribute to nitrification in aquatic systems and utilize ammonium ion as the nitrogen source for its growth under aerobic conditions. *B. subtilis* was involved largely in nitrification (Kim, Joo Park, Sook Cho, Nam, Park & Bajpai 2005) and Ghosh *et al.* (2008) inoculated *B. subtilis* in rearing tanks of ornamental fish and reported heterotrophic Bacilli was involved in the nitrification process and converted organic matter such as excreta of fish, remaining food substance to nitrate and phosphate and increased nitrate levels in the bioremediated tanks. Kim, Joo Park, Sook Cho, Nam, Park & Bajpai (2005); Queiroz & Boyd (1998) and Prabhu, Nazar, Rajagopal & Khan (1999), according to their findings, reported decreased ammonia levels, converting it to nitrate by using bioremediators. The metabolic pathways of heterotrophic bacillus strains were less complex than autotrophs (Kim *et al.* 2005) and *Bacillus* species could accomplish anaerobic dissimilatory reduction of nitrate to ammonia via nitrite (Tiedje 1988). Jafaryan *et al.* (2011) indicated that the addition of probiotic bacilli at level 1×10⁶ CFU.L⁻¹ to rearing tanks water had positive effects on the growth parameters of grass carp and might be due either to an effect on animal health or improved water quality by their action. In contrast with our results Boyd *et al.* (1984) reported that the addition of commercial probiotic bacteria had no significant effect on water quality parameters. Sharma & Bukhar (2000) indicated that the addition of Aquazyn-TM-1000, a probiotic, had no significant difference on the water quality of *Cyprinus carpio var. communis.*

Water turbidity decreased. The water transparency was also improved by macromolecular degradation of microorganisms and improved by both the decomposition and natural sedimentation of *B. cereus* (Zhao *et al.* 2009). Investigation of bacterial total count in water showed that the total bacteria in experimental treatment significantly decreased compared to blank treatment. The reason for this was the competition between bacterial flora and inoculated *Bacillus sp.* (Zhao *et al.* 2009). Moriarty (1998) and Verschuere, Rombaut, Sorgeloos & verstraete (2000) suggested that bacilli are able to use nutrients and space more than other bacteria and out-compete them by making antibiotics. Bacilli spatter many exoenzymes that can reduce slime and biofilms
around gram-negative bacteria and influence via this layer (Zhao et al. 2009). Finally, the population of gram-negative bacteria decreases. Ali (2000) and Makridis et al. (2001) encountered lower counts of Aeromonas and other gram-negative bacteria in water of fish cultured by administration of probiotic bacterial cells.

**Conclusion**

In brief, Bacillus strains play important roles in the costly nitrification–denitrification processes and have some economic advantages (Kim et al. 2005).

The present results indicate that the inoculation of probiotic bacilli at concentrations of $0, 10^6, 10^7, 10^8$ CFU/L resulted in bioremediation of effluent or waste water of fish cultivation system. The inoculation of $10^8$ CFU/L of the bacterium resulted in the highest bioremediated rate, hence $10^8$ CFU/L of the bacillus probiotics was selected as the optimal amount of inoculum and the best time of performance of this dose was 72 h.

**References**


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اثر غلظت‌های مختلف پاسیلوس‌های مفيد بر اصلاح زیستی پساب سیستم پرورش ماهی (Cyprinus carpio)
کیور معمولی
مهم‌ترین سامانه‌ای حجت الله جعفریان، حسین قلی پور‌کنعانی، محمد هریسی، محمد فرهنگی
*دانشجوی دکتری تخصصی بهداشت آبزیان، دانشگاه دامپزشکی، دانشگاه تهران
*گروه شیلات، دانشگاه کشاورزی و منابع طبیعی گیتک کاوهس
چکیده:
مطالعه‌ای از روی بررسی اثر سوء‌های اصلاح‌گره باکتریایی پاسیلوس لیچی فردر، پاسیلوس سانتیوس، پاسیلوس بلی-میکسا، پاسیلوس لاتروسرپوس و پاسیلوس سیرکولاس آتروشنکه این آکواریوم‌ها بر تنظیم برخی پارامترهای کیفی آب اعم از این آمونیاک، کلر، نیترات و کدک‌های انگریزی. پس از بررسی سیستم پرورش ماهی کیور معمولی در این آزمایش مورد استفاده کیور و آزمایش اثر باکتریایی گروه با میزان ۱×۱۰۶، ۱×۱۰۷ و ۱×۱۰۸ باکتریالی با هر لیتر از پساب در مخازن پرورش ماهی باکتریایی اضافه شد و نتایج آن مورد نظر در فواصل ۲۴، ۴۸ و ۷۲ ساعت مورد بررسی قرار گرفت. اثر زمان و غلظت در این طرح کامل‌گیری خرد شده در زمان مطالعه شد. نتایج پژوهش حاضر نشان داد که زمان‌ها و غلظت‌های متفاوت باکتریایی می‌توانند پارامترهای کیفی آب را تحت تأثیر قرار گذاردند. زمان و غلظت و اثر متقابل آن‌ها تفاوت معنی‌داری (P<0.05) بر تغییرات ایزوآمونیاک کل اثر نیترات اثر نیترات و کدک‌های داشت. شمارش مجموع باکتری‌های موجود در آب مخازن اصلاح شده ۶۲ کمتر از مخازن کنترل بود. هدف از این آزمایش، یافتن بهترین غلظت از باکتری و بهترین زمان کاربرد آن‌ها جهت کمک به تغییر پارامترهای کیفی آب در مخازن پرورش به عنوان محدود زندگی ماهی‌ها، برای افزایش رشد و بارز‌اندگی، اصلاح زیستی پساب، استفاده مجدد از منابع آب و جلوگیری از آلودگی‌های ناشی بود.
کلمات کلیدی: پاسیلوس، درمان باکتریایی، پارامترهای کیفی آب، اصلاح زیستی