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EVALUATION OF CULTURABLE, NITRIFYING, AMMONIUM-OXIDIZING, AND METABOLICALLY ACTIVE BACTERIA IN SHRIMP FARM EFFLUENTS OF KINO BAY, SONORA, MÉXICO

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Abstract - Bacteria from shrimp farm effluents in Kino Bay, Sonora, México were evaluated during the 2008 production cycle. The culturable bacterial populations considered were viable heterotrophic bacteria (VHB) and *Vibrio*-like bacteria (VLB). In addition, total bacteria (TB), metabolically active bacteria (MAB), nitrifying bacteria (NB), and ammonium-oxidizing bacteria (AOB) were quantified by epifluorescence microscopy. Three sampling sites were established in the influence area of drain and one control site far from the discharge area. Average concentrations of VHB and VLB were 10^3 and 10^2 CFU mL^{-1}, respectively. The TB ranged from 10^7 to 10^9 cells mL^{-1}. The mean values of MAB related to TB counts in the affected area were higher throughout the culture cycle compared to the control site, ranging from 1.09 to 27.35%. These results indicate that effluents modify the natural loads of bacteria in the discharge area, which could affect natural populations and the microbial balance of the area.

Key words: Ammonia-oxidizing bacteria, total and culturable bacteria, fluorescence *in situ* hybridization, oligonucleotide probe, effluents culture shrimp

INTRODUCTION

In Mexico, shrimp culture has had a great development since the late 1980's, particularly in the northwest region, i.e. the State of Sonora, the first national producer. This activity generates a large amount of effluents rich in nutrients mainly coming from unconsumed food, feces, and the metabolic excretion of the farmed organism. These effluents are discharged into different receiving ecosystems causing different impacts such as eutrophication and nutriphication (Páez-Osuna, 2001). The severity of the problem depends on the species and type of culture, as well as the management practices, especially the feeding strategies used (Casillas-Hernández et al., 2007, Martínez-Córdova et al., 2009).

Aquatic environments have the inherent capacity of self-purification through the action of bacterial populations inhabiting the water column and sediments (Jarpa et al., 2007). In this context, the water bodies can accept a moderate amount of untreated wastewaters without significant deterioration of the environment (López-López and Zaballos 2005; Atlas and Bartha, 2006).

Bacteria are naturally responsible for maintaining the balance of nitrogen compounds introduced by the effluents in the discharge areas through the process of re-mineralization and nutrient recycling (Moriarty, 1997).

Ammonium-oxidizing and nitrifying bacteria are chemolithotrophic microorganisms that use these compounds as energy sources for their development through the processes of ammonification and nitrification (Burford and Williams, 2001, Burford et al., 2003). Their important role in these processes has

been well documented in natural oceanic environments (Jetten, 2008), as well as near discharges from anthropogenic and industrial origin (Coskuner et al., 2005, Páez-Osuna 2005).

In aquaculture, scarce information about the nitrification processes is available, indicating the necessity to conduct investigations related to this important process (Hagopian and Riley 1998, Hargreaves 1998, Burford et al., 2003). The methods for studying natural populations of nitrifying bacteria are limited due to the particular characteristics of their metabolism, and long generation times; this fact limits study by traditional methods (Moriarty 1997, Daimes and Wagner 2007). The most probable number (MPN) and the plate count are the most reported methods (Fry 1998). However, in some cases, the bacterial population is underestimated because of the conditions of the methodology (Rowe et al., 1997, Watson et al., 1977) and they do not discriminate at species level the ammonia-oxidizing bacteria. By contrast, molecular methods have raised the study of metabolically active bacteria by using oligonucleotide probes directed at 16S rRNA (Mobarry et al., 1996, Coskuner et al., 2005, Daimes et al., 2001). From these molecular methods, fluorescence *in situ* hybridization (FISH) is one of the most documented because it allows the phylogenetic identification of bacteria in marine environments without previous culture (Amann et al., 1995, Pernthaler et al., 2001, Coskuner et al., 2005, García and Olmos 2007, López-Torres and Lizárraga-Partida 2007, Otoshi et al., 2009).

The aim of this research was to determine the population abundance of specific bacterial groups of ecological and sanitary importance present in shrimp farm effluents and in their influence in the discharge area.

MATERIALS AND METHODS

Study site

This research was carried out in the drain discharge of an Aquaculture Park located in Kino Bay, Sonora, México. The discharge of the drain is located 1.5 km south of the Santa Cruz lagoon mouth and carries effluents from four shrimp farms. These farms account for about 1,350 hectares of water surface with a daily exchange rate from 6 to 25% that fluctuates, depending on the culture age (COSAES 2010, D.O.F. 2008). This activity generates, approximately, 40 m^3s^{-1} of effluents at the end of the farming period (Barraza-Guardado, personal communication).

Three strategic sampling points were established in the drain and one as a control in the Bay, without apparent influence of the discharges. Site one (P1) was located at the beginning of the main drain at a junction of the drains of two farms (latitude 28º 44' 24.64" N, longitude 111º 53' 02.93" W. Site two (P2) was on the drain, 150 m before discharge into the Bay (latitude 28º 46' 17.32" N, longitude 111º 54' 23.34" W). Site three (P3) was located in the discharge 120 m perpendicular to the coastline (latitude 28º 46' 20.98" N, longitude 111º 54' 57.26" W), and site four (P4) or the control site, was 250 m southeast of Alcatraz Island (latitude 28º 48' 34.26" N, longitude 111º 57' 23.19" W) (Fig. 1).

Sampling

Four samplings were done during the production cycle of 2008: June 19th (M1), September 25th (M2), October 14th (M3), October 29th (M4); additionally a fifth sampling was done on February 5th, 2009 (M5), when no effluents were discharged from the shrimp farm. In total, 18 samples were collected from subsurface water under aseptic conditions in the sites, and dates were established.

Water samples were collected in sterile bags (Wirl Pack, NASCO) of 125 mL, which were transported to the laboratory at low temperature (4-7°C) and protected from light, to be processed immediately for culturable bacteria.

Once the culturable bacteria were inoculated, aliquots were taken from the same samples in sterile Nalgene tubes of 50 mL and fixed with filtered formaldehyde (6% final concentration, Sigma-Aldrich,

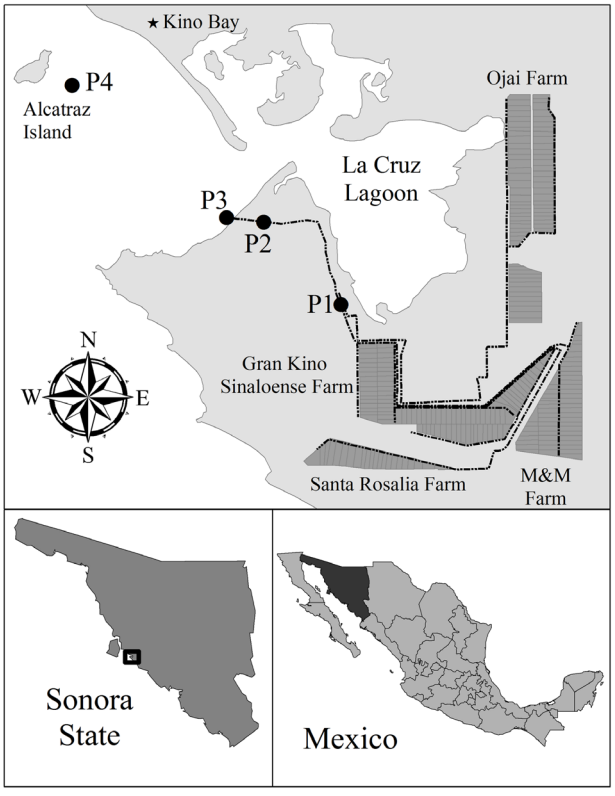

Fig. 1. Locations of La Cruz Lagoon in Kino Bay, Sonora (down left), region of Northwest México (down right) and sampling stations in collector drain effluent: P1: beginning of the drain, P2: 150 m before the discharge of drain, P3: discharge into the bay, P4: Alcatraz Island.

0.22 µm-pore-size Millipore filter) to quantify the bacteria by epifluorescence. These samples were refrigerated until processed.

Culturable bacteria

Quantification of viable heterotrophic bacteria (VHB) and Vibrio-like bacteria (VLB)

For quantification of VHB and VLB, the spread plate method (Neufeld 1985, Clesieri et al., 1998) in Marine agar 2216 and Thiosulfate-citrate-bile-sucrose agar (TCBS, DIFCO, U.S.A.) supplemented with 2% NaCl (Merck), was used. 10-fold dilution series were prepared with 3.0% of sterile seawater diluted with distilled water. The plates were incubated for 48±2 h to 30±2°C and the bacterial colonies were counted and reported as colony-forming units for mL^{-1} (CFU mL^{-1}).

Total bacteria (TB): sample preparation

A sample of 10 mL of effluent was concentrated by centrifugation at 6000 rmp (National Labnet Co. Woodbrige, N.J.) for 10 min at a volume of 0.5 mL, followed by a double-washed process with Phosphate Buffered Saline (PBS) (final buffer concentration of 10 mM phosphate and 130 mM NaCL, pH 7.2). Thereafter, the pellet was resuspended to the final volume (0.5 mL) in a 1:1 solution of PBS and ice-cold absolute ethanol (4°C). Under these conditions, the samples were refrigerated until analysis (López-Torres and Lizárraga-Partida 2007, Valenzuela-Salcedo 2010).

TB quantification

Total bacterial counts (TB) were done by epifluorescence microscopy on Teflon-coated glass slides with wells of 6 mm diameter (Precision, USA). These slides were previously coated by immersion in a warm solution (70°C) of bovine skin gelatin at 0.1% (Sigma-Aldrich) and chromium (III) potassium sulfate at 0.01% (Sigma-Aldrich). Subsequently, 6 µL of fixed cells were spotted in duplicate in each well, and dried at room temperature. The sample was previously sonicated at 70 W, 3 x 1 min (Ultrasonic cleaner, Model 1510R-MTH, Branson Ultrasonic Co. U.S.A.). These dried samples on slides were dehydrated in ethanol series to 50%, 80% and 98% (Jalmex, México) for three min each. Once they were dried, each well was treated with 5 µL of acridine orange at 0.1% (Sigma-Aldrich, USA) (Parson et al., 1984) for 5 min in darkness and later washed with sterile distilled water and filtered (0.2-µm-pore-size filter, Millipore filter). Subsequently, 4 µL of immersion oil were added over each well (type A, refractive index = 1.5150, Jalmek, Mexico) and 20 fields, chosen randomly, were counted by duplicate, in an epifluorescence microscope (Labomed Model CRLC2, U.S.A) equipped with excitation and emission filters of 420-480 nm.

The total number of cells mL^{-1} ($\boldsymbol{A}$) was calculated according to Oliveira et al. (2003), and Vargas-Cárdenas and Lizárraga-Partida (1999) as:

$$A = \frac{\left(\frac{b}{c}\right) f}{d}$$

where $\boldsymbol{b}$ is the number of cells counted, $\boldsymbol{c}$ is the number of the microscopy fields counted, $\boldsymbol{f}$ is a conversion factor (ratio of well area of the glass slides and microscopy field area counted), and $\boldsymbol{d}$ is a dilution factor (volume used x dilution).

Quantification of metabolically active bacteria

The metabolically active bacteria (MAB) were quantified by fluorescence *in situ* hybridization (FISH), using the EUB338 probe (Eubacteria) according to the methodology described by López-Torres and Lizárraga-Partida (2007), and modified by ourselves. The samples were prepared similarly to that for BT before the addition of acridine orange.

Ammonium-oxidizing bacteria

The evaluations were made using the probes Nsm156 (β-*Proteobacteria; Nitrosomonas* spp.), Nsv443 (β-*Proteobacteria*; *Nitrosolobus multiformis, Nitrosospira briensis* and *Nitrosovibrio tenuis*) (Mobarry et al., 1996, Coskuner et al., 2005), plus NIT3 (*α-Proteobacteria*; *Nitrobacter* spp.) (Wagner et al., 1996). These probes, as well as EUB338, were fluorescently labeled in the 5' position with the fluorochrome 5-Carboxytetramethylrhodamine (5-TAMRA) during its synthesis by Genosys-Sigma (México).

One µL of the probe (3 ηg µL-1) of the oligonucleotide was added to each sample-containing well, followed by 8 µL of hybridization buffer (the final concentrations were 0.9 M NaCl, 0.01% sodium dodecyl sulphate (SDS), 20 mM TRIS-HCL, pH 7.2 and 35% Formamide (Sigma-Aldrich)). The hybridization conditions were according to those recommended by López-Torres and Lizárraga-Partida (2007), using a bacteriological incubator (Stabil-Therm, U.S.A), for 12 ± 1 h at temperature of 46±0.5ºC.

Microscopic analysis and the counting of hybridized cells were done under the conditions described above for TB, but using CITIFLUOR AF87 (Hartfield, PA, U.S.A) as a mounting solution to avoid bleaching of the sample, plus a low fluorescence immersion oil. Data MAB hybridized cells were calculated as a percentage of TB counted with acridine orange. For Nsm156, Nsv443 and NIT3 probes, the percentage of hybridization was calculated as a percentage in relation to the MAB.

Statistical analysis

The software ProStat version 3.0 was used for statistical analysis. Data on the number of bacterial counts were transformed to Log_{10} before analysis, as recommended by Zar (1984). Analysis of variance (ANOVA) with a confidence interval of 95% was performed. To determine significant differences among the sampling points and/or between the test samples, a Fisher test of multiple range least significant difference (LSD) was applied.

RESULTS

Culturable bacteria: VHB and VLB

The abundance of the bacteria counted in the five samplings is shown in Fig. 2. A similar abundance of VHB was found in the three sites of the drain. The highest value was found in P1 with 2.16×10^4 CFU mL^{-1}, in the first sampling (M1), while the lowest of 9.0×10^1 CFU mL^{-1} was observed in P4 during the second sampling (M2) (Fig. 2). The average for the first 4 sampling and points was $9.07 \pm 7.35 \times 10^3$. Statistical analysis showed no significant differences on the microbial load among the three drain sites, but differences were observed in comparison to the control (P4).

VLB counting was always around 10^2 CFU mL^{-1}, except for the fourth sampling (M4) when 1.24×10^3 was reached in P3, while a concentration of 2.5 x

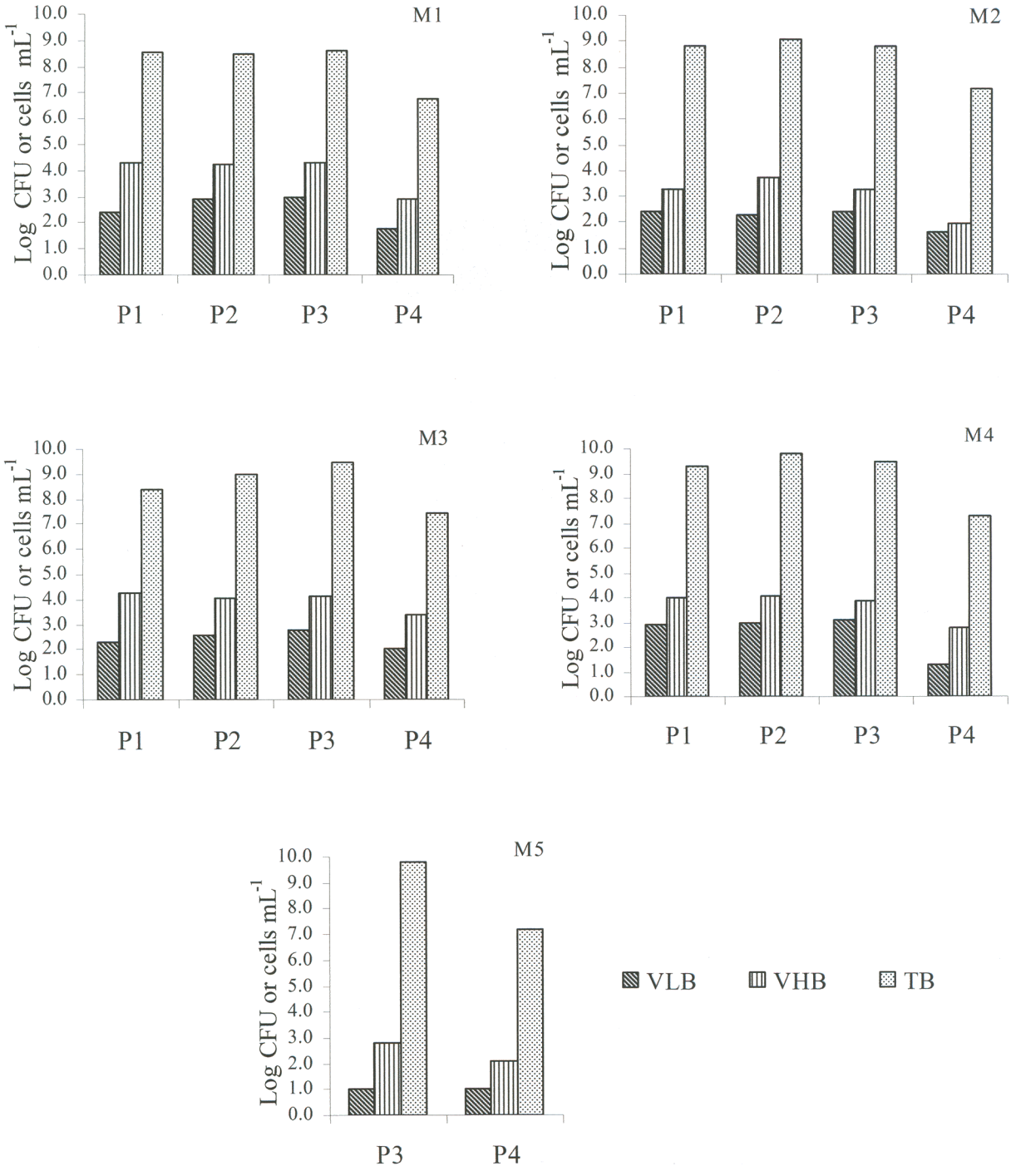

Fig. 2. *Vibrio*-like bacteria (VLB), viable heterotrophic bacteria (VHB) and total bacteria (TB) determined during five sampling (M1, M2, M3, M4, M5) performed in the drain discharge (P1, P2, P3) of shrimp farms and Alcatraz island (P4) in Kino Bay, Sonora, México.

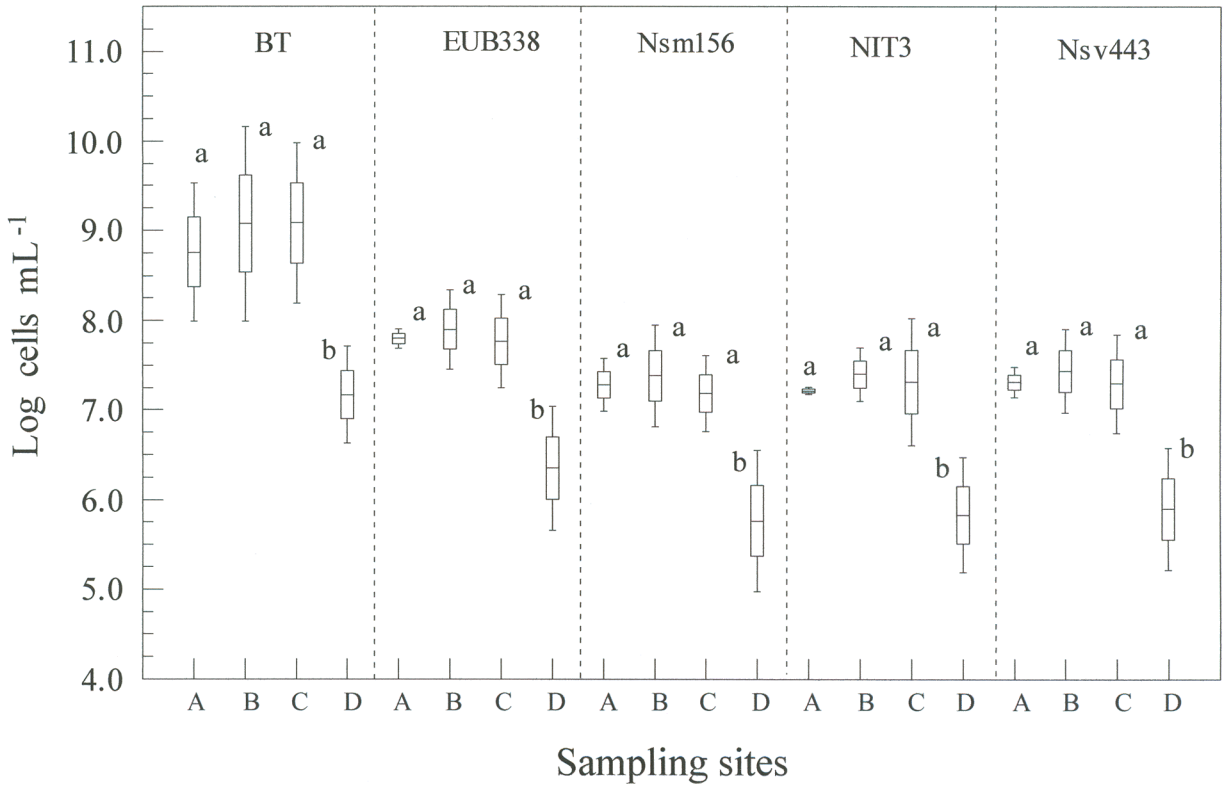

Fig. 3. Average abundance of total bacteria (TB), metabolically active bacteria (MAB): Domain Eubacteria (EUB338), *Nitrosomonas* spp. (Nsm156), *Nitrobacter* spp. (NIT3), *Nitrosospira* spp. (Nsv443) in the sampling sites (A:P1, B:P2, C:P3, D:P4), in effluents from shrimp farming in Kino Bay, Sonora, México. Different letters represent statistical differences among groups of bacteria ($P < 0.05$).

10^1 CFU mL^{-1} was found in P4 in the same sampling (Fig. 2). The average of VLB was 4.48 ± 3.82 x 10^2 cells mL^{-1}. No significant differences were found in the microbial load of the drain sites, but differences were detected when compared with the control point (P4).

In the fifth sampling (M5) without effluent discharge, VHB counting was 6.0 x 10^2 CFU mL^{-1} in P3 and 1.25 x 10^2 CFU mL^{-1} in P4, while the VLB counting was 1.0 x 10^1 CFU mL^{-1} for both sites (Fig. 2).

Total bacteria

The TB ranged from 6.08 x 10^6 cells mL^{-1} in P4 during the first sampling (M1) to 6.33 x 10^9 cells mL^{-1} in P2 during the fourth sampling (M4) (Fig. 2). The overall average was 1.17 ± 1.19 x 10^9 cells mL^{-1}. No significant differences among the microbial loads of the drain sites were found, but differences were detected in comparison to the control point.

In the sampling without effluents, the concentrations of TB in sites P3 and P4 were 6.01 x 10^9 cells mL^{-1} and 1.63 x 10^7 cells mL-1, respectively.

Bacteria quantification with specific probes

Probe EUB338: Domain Eubacteria

The abundance of this bacterial group with respect to the BT determined by epifluorescence during the entire shrimp culture cycle is shown in Fig. 3. The quantified Eubacteria with probe EUB338 (MAB) ranged from a minimum of 9.59 x 10^5 cells mL^{-1} in P4 of the first sampling (M1) to a maximum of 1.42

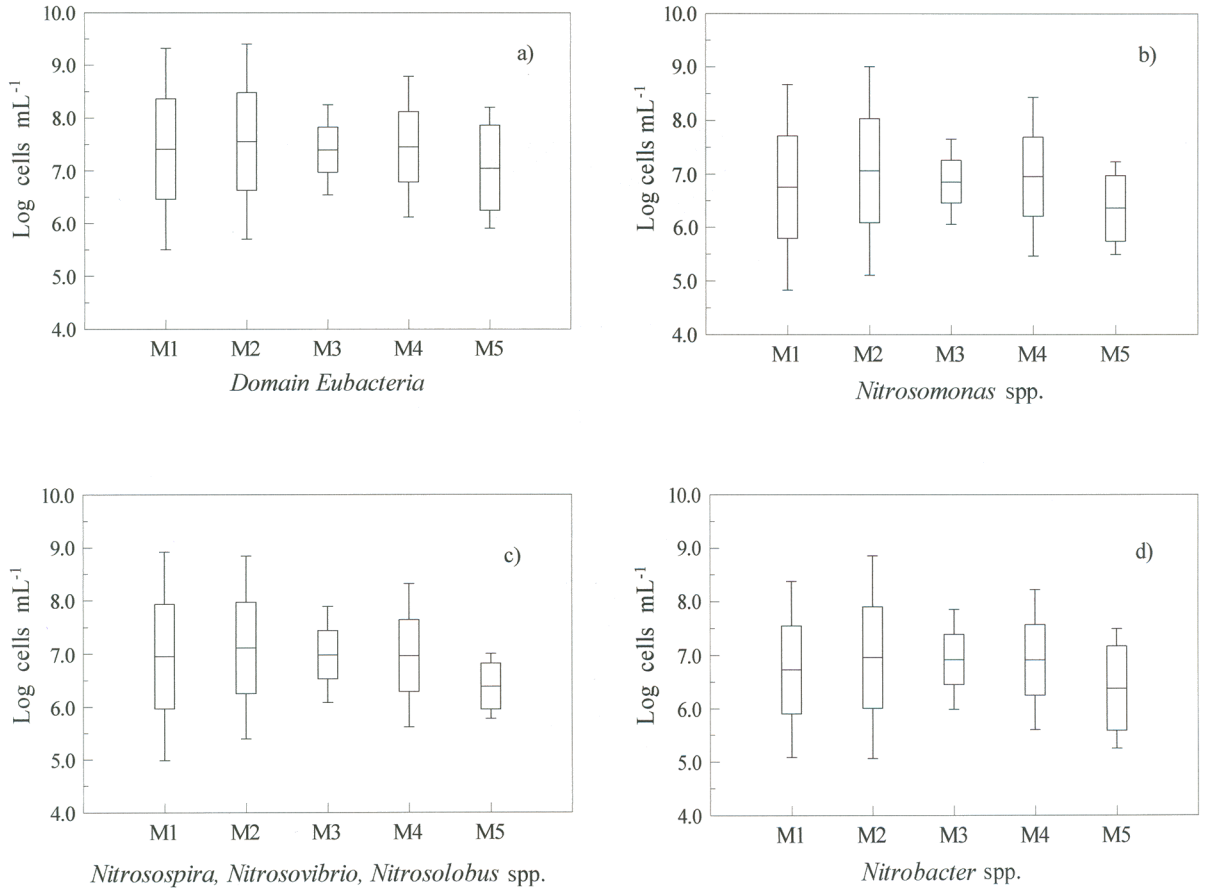

Fig. 4. Average abundance of metabolically active bacteria (MAB) recording in the discharge drain during the 2008 culture cycle in Kino Bay, Sonora, México. a) EUB338 probe, b) Nsm156 probe, c) Nsv443 probe and d) NIT3 probe. There was no statistical difference between samplings.

x 10^8 cells mL^{-1} in P2 of the second sampling. The global average of the MAB was 5.48 ± 4.11 x 10^7 cells mL^{-1}. Statistical analysis did not show significant difference among the three analyzed drain points, but differences were found when compared to the control (P4).

In the sampling without effluents (M5), an abundance of 2.87 x 10^7 cells mL^{-1} was found in P3, a value lower than the average found in MAB, although in the same order of magnitude. By contrast, in P4 an abundance of 4.43 x 10^6 cells mL^{-1} was found, a value above the overall average found in this sampling point for this same bacteria group (Fig. 4a).

Ammonium-oxidizing bacteria

Probe Nsm156: subclass β-Proteobacteria; Nitrosomonas spp.

The quantification of this group of AOB with the Nsm156 probe showed abundance in the discharge drain that ranged from 5.02 x 10^7 cells mL^{-1} in P2 of the second sampling, to 2.14 x 10^5 cells mL^{-1} in P4 of the first sampling. The overall average in this bacteria group was 1.74 ± 1.42 x 10^7 cells mL^{-1} (Fig. 3). No significant differences among the bacterial abundances of the three drain sites were found, but differences were observed when compared to the control.

In the last sampling without effluent discharge (M5), the abundance of *Nitrosomonas* spp. in P3 and P4 was lower, 4.62 x 10^6 and 1.13 x 10^6 cells mL^{-1}, respectively (Fig. 4b).

Probe Nsv443: subclass β-Proteobacteria; Nitrosolobus multiformis, Nitrosospira briensis and Nitrosovibrio tenuis

The oligonucleotide probe Nsv443 of the subclass β-Proteobacteria showed a bacterial abundance of this AOB ranging from a maximum of 5.35 x 10^7 cells mL^{-1} in P2 of the second sampling to a minimum of 3.0 x 10^5 cells mL^{-1} in P4 in the first sampling. The overall mean was of 1.87 ± 1.48 x 10^7 cells mL^{-1} (Fig. 3). No significant differences in the abundance of *Nitrosospira* spp. in the three discharge points analyzed were found when compared to the matching control: difference was found when compared to control in P4.

In the last sampling without effluent discharge, the abundance of this bacterial group in P3 and P4 was 3.99 x 10^6 and 1.47 x 10^6 cells mL^{-1}, respectively (Fig. 4c).

Probe NIT3: subclass α-Proteobacteria Nitrobacter spp.

The quantification of the BN group with the NIT3 probe showed an abundance of *Nitrobacter* spp. that ranged from 4.96 x 10^7 cells mL^{-1} in P3 of the first sampling to 3.60 x 10^5 cells mL^{-1} in P4, also in the first sampling (Fig. 3). The overall average of *Nitrobacter* spp. was 1.64 ± 1.37 x 10^7 cells mL^{-1}. No significant difference was found between the three sites analyzed, but differences were found when compared to the control (P4)

In P3 and P4 of the last sampling (M5), an abundance of 5.89 x 10^6 and 9.48 x 10^5 cells mL^{-1}, respectively, was found (Fig. 4d).

The average abundance and behavior of all groups of bacteria during the shrimp culture cycle are shown in Fig. 4. In general, there was a tendency of increase in the first two samplings. Later, the values were similar in the third and fourth samplings, although with a lower abundance. During the last sampling, with no discharge of effluents, a strong decrease in the bacterial abundance was recorded. However, no significant difference was found among samplings done in the bacterial groups studied.

Fig. 5 shows the average hybridization percentages related to the TB determined with bacterial probes in the three sites of influence of the drain (P1-P3) and Alcatraz Island (P4). In general, the lowest averages, except for the Nsv443 probe, were found in P3 with 1.88% for the NIT3 probe and 2.17% for the Nsm156 probe. The highest percentages were found in P4 with values of 4.23%, 4.98% and 5.50% for Nsm156, NIT3 and Nsv443, respectively.

In MAB (EUB338 probe), the percentage of hybridization in relation to TB ranged from 1.09 % (P3, M3) to 27.35% (P2, M1) during the shrimp culture cycle. In P3 the lowest percentage of hybridization of the MAB was found with 0.48%, which corresponded to the last sampling (M5), in which there was no effluent discharge. In this same sampling, in P4 a hybridization percentage of 27.20% was found very similar in value to the highest found in P2 (27.35%) for this same group of bacteria.

Fig. 6 shows the hybridization percentages of bacteria groups with the different oligonucleotide probes (with respect to the MAB, probe EUB338) and samplings done. The percentages of ammonium-oxidizing bacteria and nitrifying bacteria remained in a more o less constant percentage in the different study points, without a defined trend, except for the alternating of the bacterial groups in the different drain points. With the Nsm156 probe, it a hybridization percentage in the drain discharge was found for *Nitrosomonas* spp., which ranged from 14.05% (M1, P3) to 40.29% (M2, P1) (Figs. 6a, 6b). The average percentage of hybridization for this group of bacteria during the shrimp culture cycle was 28.29%.

With the Nsv443 probe, a hybridization percentage of *Nitrosospira* spp., ranging from 29.90% (M3,

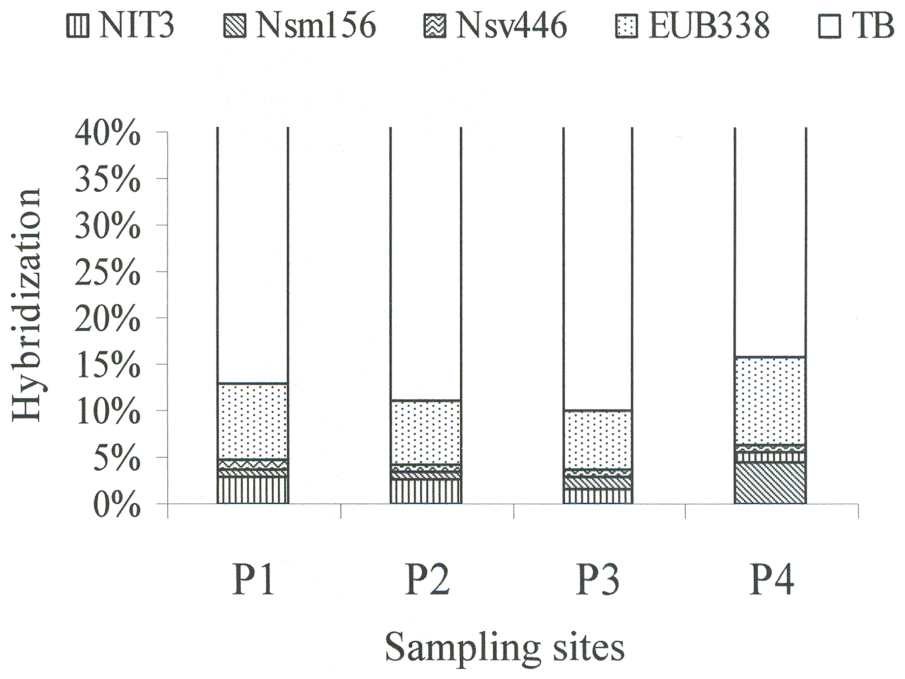

Fig. 5. Average percentage of hybridization determined with oligonucleotide probes; Nsm156, Nsv443, and NIT3 related with TB in the monitoring sites: P1: begining of the drain, P2: 150 m before discharge of the drain, P3: discharge into the bay, P4: Alcatraz Island.

P1) to 46.19% (M2, P4) was found in the discharge drain (Figs. 6c and 6b). For this group of AOB, the highest average percentages in the study, 32.75%, 34.48%, 33.91% and 35.69%, were found in P1, P2, P3 and P4, respectively, when the drain was in operation, denoting greater bacterial abundance in the studied effluents.

The quantification of the NB group with the NIT3 probe, showed a lower hybridization percentage of *Nitrobacter* spp., which varied from 5.98% (M1, P3) to 39.81% (M1, P4) (Fig. 6a). The average hybridization percentage of *Nitrobacter* spp. was 28.4%.

During the non-activity stage of the shrimp farms (M5), it was found in the discharge drain (P3) that the hybridization percentages related with MAB were lower for the three probes, with 16.11% for Nsm156, 13.91% for Nsv446 and 20.53% for NIT3. Contrarily, for P4 in the same sampling, higher percentages of hybridization (25.41% for Nsm156, 33.23% for Nsv446 and 21.4% for NIT3) were found, which indicates a great bacteria activity in the control site.

DISCUSSION

The study of the diversity of marine bacteria is important for understanding the structure of communities and their distribution patterns in their different habitats. The marine environment is characterized by hostile parameters such as high pressure, fluctuations in salinity and temperature, absence of light and others. However, bacteria in general are adapted to survive under different environmental conditions (Das et al., 2006).

The effluents of shrimp farms are rich in organic load and inorganic nitrogen compounds mainly from unconsumed or undigested food, dead organisms, metabolites, bacteria and others (Burford et al., 2003, Páez-Ozuna 2005, Martínez-Córdova et al., 2009). In aquatic environments, bacteria are responsible for metabolizing these compounds and incorporat-

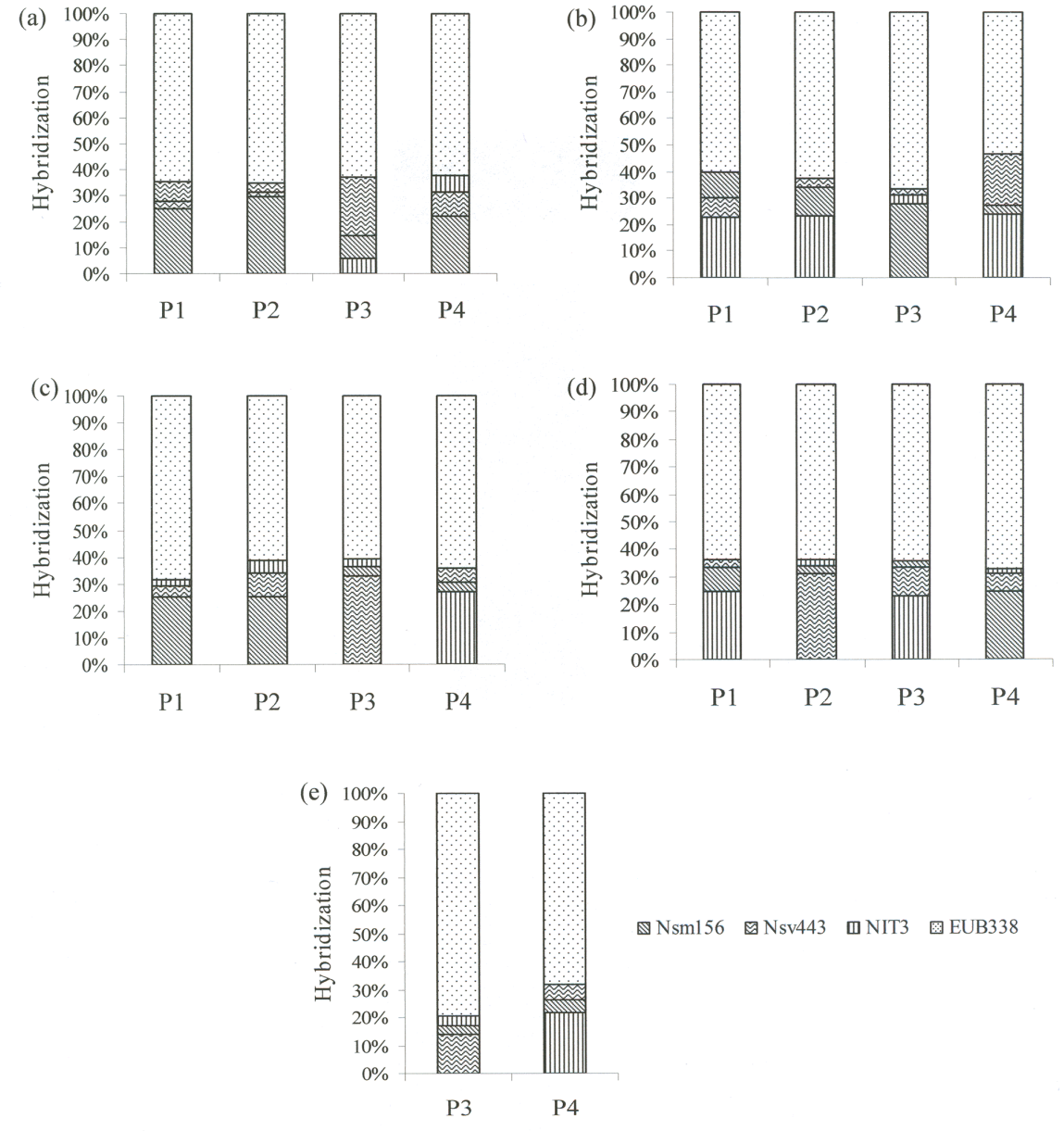

Fig. 6. Percentages of hybridization determined with oligonucleotide probes Nsm156, Nsv443, and NIT3 related to BMA (EUB338) in the sites monitored: P1: begining of the drain, P2: 150 m before the discharge of the drain, P3: discharge into the bay, P4: Alcatraz Island. Samplings: a) M1, b) M2, c) M3, d) M4, e) M5.

ing them into the food chain (Burford and Williams 2001, Burford et al., 2003). The concentration, type and distribution of microorganisms *in situ* is strongly related to their ecological function and competitive success, being strongly influenced by abiotic factors such as the spatial structure of the habitat, temperature and nutrient availability (Daimes and Wagner 2007).

In this regard, the average loads of VHB and VLB found in drain effluents during this study agree with the values published by Chomina-Huérigo and

Haros-Méndez (2008) in a study in the Santa Cruz Lagoon at sites P1 and P3 (same location as for this study). This indicates that the culturable bacteria levels, at the time when the drain is operating, have not changed significantly in comparison to previous years. In this sense, although the bacterial loads of VHB and VLB found in the present study are not lethal for marine organisms in the region, when the effluents are constantly discharged they affect the area of influence with other microbial populations different to the normal microbiota of the ecosystem; therefore, substantial changes are not discarded in the balance of natural populations.

In this regard, Chomina-Huérigo and Haros-Méndez (2008) reported a high incidence of *Vibrio vulnificus* (71% of isolated bacteria) in shrimp effluents discharged into the Santa Cruz lagoon where bivalve mollusks are cultivated. The bivalve can concentrate these microorganisms in their tissues and cause their mortality or public health problems when the oysters are consumed raw (Linkous and Oliver 1999, Torres-Alvarado and Calva-B. 2004). However, during the time when no effluents were discharged, VHB concentrations detected in the M5 were between 1 and 2 orders of magnitude lower than those found when the drain was operating. For the VLB, the loads found decreased one order of magnitude and showed a similar concentration in the discharge area and the Alcatraz Island. This indicates that the system is able to dump the bacterial loads discharged in the effluents.

Scientific information regarding the TB in effluents is scarce (mostly data reports of water or sediments of shrimp culture ponds). Otoshi et al. (2009) reported values of TB of 3.9 x 10^8 cells mL^{-1} in eutrophic environments of super-intensive culture of *L. vannamei* (400 shrimps m^{-2}), similar to those found in this study in the three drain sites. In contrast, Burford et al. (2003) reported TB values from 3.35 to 5.42 x 10^7 cells mL^{-1} in a shrimp culture recirculating system in Belize. Similarly, Fernandes et al. (2010) reported for *Penaeus monodon* culture TB values from 2.8-3.4 x 10^5 to 9.65 x 10^6 cells mL^{-1} close to those found in P4 of the present study. Both studies reported TB concentrations up to two orders of magnitude beneath the ones found in the effluent, which indicate the potential microbial impact in that area.

The concentration of TB in P3 during the last sampling without effluent discharge (6.1 x 10^9 cells mL^{-1}) was the same as that found in the fourth sampling (6.33 x 10^9 cells mL^{-1}), indicating that the concentration did not decrease after discharge was suspended. In contrast, the culturable bacteria abundance was significantly lower when no effluents were discharged. It could be inferred that the high concentrations of BT could be due to the re-suspension of nonculturable bacteria from sediments caused by wind.

The highest values of TB were found in P2, where more turbulence generated by the orography of the channel was observed, which coincides with the hypothesis of re-suspension of sediment material. The lowest average values of BT were found in P1 which corresponds to the beginning of the drain. The same trend was observed with culturable bacteria.

According to Primavera (2006), the effluents can drag about 45% Nitrogen and 22% of organic matter during the exchanges and the harvest time that is deposited as sediment in the receiving water body. In aquatic environments, the reincorporation of nutrients into the water column is made by the MAB, including some heterotrophic bacteria, chemolithotrophic ammonium-oxidizing, nitrifying, and other bacteria, which are responsible of the nitrification process (Moriarty 1997, Hagopian and Riley 1998, Burford and Williams 2001, Ward 2008).

In this study, the percentages of hybridization of MAB with respect to TB in the three drain sites were lower than the range reported by López-Torres and Lizárraga-Partida (2007) in water and biofilms of larval rearing of *L. vannamei* (27 to 70%). This difference was probably because the biofilms allow the colonization of a great diversity of organisms (Ivanov et al., 2006) in contrast to the water column, where bacteria remain in suspension or

sediment on particulate matter. In the same way, this differs with Jorquera et al. (2004) who found a percentage of hybridization ranging from 38% to 58% for Eubacteria in commercial cultures of *Argopecten purpuratus*.

Valenzuela-Salcedo (2010) found in a bioremediation system of *L. vannamei* effluents hybridization percentages from 20 to 30%, near to the maximum value found in this study.

Nitrifying bacteria have a great ecological importance because they convert ammonium into nitrate via an intermediate step of nitrite production (Spieck and Bock 2005). These bacteria include two groups of microorganisms highly specialized in the nitrification process in different environments: ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (Sinha and Annachhatre 2007). In this study, the abundance of AOB bacteria (*Nitrosomonas* spp. and *Nitrosospira* spp.) and nitrifying bacteria (*Nitrobacter* spp.) differed in 3 and 4 orders of magnitude above those reported by Abraham et al. (2004) in studies carried out in India on the cultured shrimp *L. vannamei*. These authors reported in pond water 9.2×10^4 NMP of AOB in 100 mL of water (9.2×10^2 NMP mL^{-1}). These concentrations were lower than we found in this study by FISH with oligonucleotides probes (Nsm156 y/o Nsv443). Similarly, in India Rao and Karunasagar (2000) published values of concentrations of *Nitrosomonas* spp. in a range from <2 to 2.3×10^2 NMP L^{-1} (mean 6.5×10^1 NMP L^{-1}) and of *Nitrobacter* spp. ranging from 1.8×10^1 to 2.0×10^2 NMP L^{-1} (mean 8.4×10^1 NMP L^{-1}) in shrimp culture. These values contrast with the findings in this study and may be attributed to the methodology used to determine the bacterial abundance.

According to Amann et al. (1995), culturable marine bacteria represent a range from 0.001 to 0.1% of the total population due to their specific development requirements. In the case of ammonium-oxidizing and nitrifying bacteria, they have a limited growth in traditional culture media due to their chemolithotrophic metabolism (Spieck and Bock 2005, Koops and Pommerening-Röser 2005).

Different authors: Manz et al. (1992), Amann et al. (1995), and Mobarry et al. (1996) have suggested that FISH with specific probes directed to rRNA is the choice methodology to determine the exact number of existing microbial cells in environmental samples. Additionally, it has the advantage that allows their identification depending on the specificity of the probe used (Amann et al., 2001). In this study, the two oligonucleotide probes used for AOB, (Nsm156 and Nsv443) obtained average values in the same magnitude order (10^7 cells mL^{-1}) in the three sites of influence of the drain without statistical difference among them. With the Nsv443 probe, a slight tendency to increase during the greatest water exchange was observed, as well as the highest hybridization percentages for this bacterial group. Some studies of the nitrification process reveal that the group of *Nitrosomonas* spp. is frequently the predominant group in close association with *Nitrobacter* spp. However, in this study, the group of *Nitrosospira* spp. was the group that showed the greatest abundance.

With regard to NB, Otoshi et al. (2009), in hyperintensive cultures of *L. vannamei*, reported the use of a Ntspa712 probe directed to the taxon *Nitrospira* (nitrite oxidant), finding concentrations of around 1.2×10^8 cells mL^{-1} which represented a 31% of hybridization in relation to TB (3.9×10^8 cells mL^{-1}). These hybridization percentages were similar to the highest percentage found in this study (37.64%) with the probe NIT3 for the group of NB. In general, the percentage of nitrifying and ammonium-oxidizer bacteria found in this study corresponds to values found in water with high concentrations of nitrogenous compounds (Urakawa et al., 2006).

Shrimp farming in our country and particularly in our State, is done in traditional farming systems with high water exchange, generating a great amount of effluents with high nitrogen loads that cause eutrophication and nutriphication in the aquatic receiving systems (Miranda et al., 2007, 2009).

According to the percentages of nitrifying and ammonium-oxidizers bacteria consistently found in this study in the discharge of the drain, we can

conclude that the transformation of these nutrients by the bacteria inside the ponds through the process of nitrification is feasible. Therefore, an alternative to consider is the use of closed culture systems with recirculation or low exchange (Piedrahita 2003, Cáñez-Figueroa 2011). These systems use different physical and biological processes or are combined to achieve appropriate culture water for reuse, closing the cycle of nitrogen (Tal et al., 2003, Crab et al., 2007). Furthermore, the closed recirculation systems have several advantages over open systems because they can control production, water quality, temperature and diseases, as well as providing the best environment for the cultivated species (Shan and Obbard 2001, Barón-Sevilla et al., 2004).

In aquaculture, there are few microbiological studies published and most of them have been focused primarily on certain groups of culturable heterotrophic microorganisms of pathological interest, such as *Vibrio* spp. for its potential to produce diseases in the shrimp farm. Future work must develop research into the groups of bacteria of ecological interest involved in the nitrification process in closed recirculation systems, to produce shrimp in environmental friendly conditions. In the same way, applying treatments of biofiltration of the water to be recirculated to promote the generation of bacterial biofilms for improvement of water quality is needed.

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