Vibrio nigripulchritudo monitoring and strain dynamics in shrimp pond sediments

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Abstract:

Aims: A description of bacterial pathogens in shrimp ponds is necessary to understand their pathogenesis. *Vibrio nigripulchritudo* was shown to contain saprophytic and pathogenic strains among New Caledonian isolates. We established a method to map the development of *V. nigripulchritudo* in pond sediments at three different genetic levels: the species level, then at the pathogenic cluster level and finally at the plasmid level, present only in all highly pathogenic isolates.

Methods and Results: PCR methods were applied to shrimp pond sediments both before and after a mortality outbreak. Using crude samples, the species *V. nigripulchritudo* is not detected at first (0/42 samples at day 56 post stocking) but appears frequently in the sediments after the mortality event (30/42 at day 107). The distribution of strains from the pathogenic cluster of *V. nigripulchritudo* also follows this pattern. In contrast, the pSFn1 virulence-associated plasmid was detected in one sample at day 56 and none at day 107. An enrichment method was developed to lower the detection limits of our assays. After enrichment, the species *V. nigripulchritudo* was detected in all samples at both dates. The number of samples positive for pSFn1 was 42/42 samples at day 56 and 29/42 at day 107.

Conclusions: These results show that the sediments contain *V. nigripulchritudo*, notably pathogenic strains. Surprisingly, the virulence-associated plasmid pSFn1 found in all *V. nigripulchritudo* isolated from moribund shrimp appears less frequently in sediments, possibly being useless or even detrimental to its recipient bacteria in this environment.

Significance and Impact of the Study: This study confirms the presence of pathogenic *V. nigripulchritudo* strains in shrimp pond sediment before a mortality outbreak complying with a previous hypothesis that sediments could be the infecting reservoir. After the outbreak, both total *V. nigripulchritudo* and pathogenic strains populations have largely increased, possibly contributing to the recurrent mortality observed in this shrimp vibriosis.

1. Introduction

In New Caledonia, located in the South Pacific Ocean, shrimp pond farming is based on the culture of the domesticated Pacific blue shrimp *Litopenaeus stylirostris*. Since 1980 production has increased from nonexistent to a total of about 2000 tons per year (*Institut de la statistique et des études économiques*, www.isee.nc). Although the production is small compared to the world shrimp production (2.6 million tons for penaeid shrimp, www.ifremer.fr/aquaculture), in New Caledonia this industry is the second export business on the island. However, shrimp aquaculture in New Caledonia can be threatened by disease outbreaks.

Two seasonal vibrioses regularly affect shrimp populations and can cause severe mortalities in specific environmental conditions (Mermoud et al., 1998; Goarant et al., 2006a). Since the use of antibiotics in ponds has been banned in New Caledonian farming procedures (Andrier, 2003), fighting against bacterial diseases solely depends on prophylaxis. Therefore, studies on the pathogenic *Vibrio* species were undertaken to find ways to decrease the risk of disease and disease spread (Goarant et al., 2006b).

An emerging cluster of highly pathogenic *Vibrio nigripulchritudo* has been identified as the etiological agent for Summer Syndrome (Goarant et al, 2006a, b). This *Vibrio* species was also recently reported in mass mortality outbreaks in *Marsupenaeus japonicus* in Japan (Sakai et al., 2007). In New Caledonia, this disease occurs during the warm season and as of 2007, it affects 3 out of 17 farms of New Caledonia and can wipe out as much as 80% of the shrimp population in a pond around day 55-60 of the 120-day grow out period (Lemonnier et al., 2006). The profitability of the farms struck by the disease is threatened by the recurrent mortalities induced by *V. nigripulchritudo*. The risk of disease outbreak depends on interdependent parameters such as environment quality (Kautsky et al., 2000; Lemonnier et al., 2006), shrimp physiology (Wabete et al., 2008) and bacterial virulence which was shown to be strain-specific within the species *V. nigripulchritudo* (Goarant et al, 2006a).

Genotype analysis of a collection of strains allowed the identification of a cluster of strains identified by AP-PCR and MLST that contains all highly pathogenic isolates (Goarant et al, 2006b). This suggested that virulence can be predicted using genetic markers. To investigate the genetic basis of virulence, subtractive hybridization (SSH) experiments were undertaken between a highly pathogenic tester strain *V. nigripulchritudo* and a non pathogenic driver strain *V. nigripulchritudo* (Reynaud et al., 2008). Sixty eight SSH fragments were detected in all virulent strains and within these 68 fragments, 13 were restricted to the most virulent strains. Of these 13 markers, 10 are localized on an 11237 bp plasmid named pSFn1 that possibly carries virulence determinants. Because it is consistently associated to high virulence in New Caledonian *V. nigripulchritudo* strains (Reynaud et al., 2008), it can be regarded as a virulence marker.

Sediments have been suggested to be a potential reservoir of these pathogenic *Vibrio* (Goarant et al. 2006a) as was already described for other Vibrios (Kaneko and Colwell, 1978), including fish or shrimp pathogens (Enger et al, 1989 ; de la Peña et al., 1992). This study aims at describing the bacterial dynamics in pond sediments by mapping total heterotrophic bacteria and *V. nigripulchritudo* at the species and strain levels with both molecular and culture-dependent techniques.

2. Materials and method

Experimental grow out and shrimp analysis

The pond used in this study covers 1520 m^2. Water was first pumped in on august 15, 2007 and a week later, postlarvae (PL) were used to stock the pond at a density of 18.2 $PL.m^{-2}$ (day 1). Water renewal and feeding followed the usual rearing procedures as described

elsewhere (Lemonnier et al., 2006) and a total of 300 kg of feed was distributed over the whole rearing period. Moribund shrimp were collected and analyzed as previously described (Goarant et al., 2006). Briefly, one drop of haemolymph aseptically withdrawn from the ventral sinus was plated on solid Marine Agar with 2% glycerol (weight/volume) and incubated at 30°C for 72 hours. Putative *V. nigripulchritudo* colonies were identified as black colonies in the presence of glycerol.

Sediment sampling and analysis

Pond sediments were collected at 42 points in staggered rows with two 50-mL core samplers. For each point, two 3-cm deep sediment cores were collected underwater and transported to the laboratory within 15 minutes.

Immediately after sampling, temperature and pH were measured using a Hanna instrument pocket thermometer and an IQ Scientific field pH meter. Sediment dry weight was determined by measuring the weight loss after drying at 60°C for 5 days in a Memmert oven. Ash free dry weight was measured for an estimation of organic matter content by burning the dried sediments for 5 hours at 550°C in the oven. Chloroplastic pigment concentration was calculated using the spectrophotometric method, after acetone extraction, from the equations of Lorenzen and Jeffrey (1980) for chlorophyll a, b and c.

Sediments were also artificially inoculated with known concentrations of *V. nigripulchritudo* as positive controls.

Bacterial strains, media and DNA extraction

Vibrio strains used in this study are listed in tables I and II. All the strains isolated in New Caledonia are deposited in the bacterial pathogen collection for marine aquaculture species (*Centre de Ressources Biologiques, Laboratoire de Génétique et Pathologie, Institut Français de Recherche pour l'exploitation de la MER, La Trembalde, France,* www.ifremer.fr/crb). DNA from bacterial Marine broth cultures was purified using the High Pure™ PCR Template Preparation kit (Roche Diagnostics). DNA from sediments was isolated using the PowerSoil™DNA Isolation Kit from MO BIO Laboratories following the manufacturer's instructions.

For total heterotrophic bacteria and presumptive *Vibrio nigripulchritudo* identification, serially diluted wet sediments were spread on solid Marine Agar supplemented with 2% (weight/volume) glycerol (Baumann and Schubert, 1984). The enrichment solution was marine broth diluted ½ with sterile artificial sea water. Sediments to be enriched (5g of wet sediments in 10 mL enrichment solution) were incubated 24 hours at 28°C with constant agitation (120 rpm). Then, DNA was extracted as described above.

PCR

Amplifications were done in an AppliedBiosystem 9700 thermal cycler for routine PCR and in a Roche LightCycler® 2.0 Carousel-Based System for real time PCR. The following primers were used for universal 16S rDNA amplification CTCAGATTCAACGCTGGCGG and GGCGGTGTGTACAAGGCCCG, for *V. nigripulchritudo* species characterization, VnF and VR with an annealing temperature of 58°C (Goarant et al., 2006a).

For plasmid pSFn1 detection, primers pSFn1-6020 and pSFn1-6974 were designed using the Primer3 software from a previously published sequence of pSFn1 (accession number EU156059, Reynaud et al., 2008) and had the following sequences pSFn1-6020 TGTCTTCTGGATCGCTTCGCC and pSFn1-6974 CGTCGTAAGGAGCGATAAGCC (annealing temperature 55°C), generating a specific 954-bp long amplicon. Reagents (polymerase and buffers) were from QIAGEN and used following the manufacturer's protocol.

The "Summer syndrome" emerging cluster of pathogenic strains was identified and quantified in sediment DNA samples using real time PCR with hybridization probes targeting a particular polymorphism in *gyrB* as previously described (Goarant et al., 2007).

Universal 16S amplification was used as an internal amplification control when PCR results were negative with the other primers.

Statistical analysis and maps

Statistical analysis including mapping was done using the S-PLUS software (Seattle, WA, USA). Bacteria means were compared using t-tests at a 5% signification threshold after log-transformation.

3. Results

General pond evolution during the study

Shrimp were harvested on December 11, 2007 after 112 days of grow out. Final survival was 58% and shrimp mean individual weight was 9.75 g. On days 89 and 95, one and 12 moribund shrimp were observed and collected for analysis respectively. *V. nigripulchritudo* septicemia was confirmed as the etiological cause of the mortality of these shrimp, all haemocultures displaying a heavy monomorphous colonization by *V. nigripulchritudo*. Typical Summer Syndrome was confirmed using the genetic tools available (AP-PCR and plasmid pSFn1-specific PCR) on cultivated isolates.

Mean chemical and physical parameters of the pond are presented in table III. During the production cycle, mean temperature increased from 23.8 °C to 29.7 °C, reaching the critical 28°C known to enhance the risks of Summer Syndrome (Lemonnier et al., 2006). The overall pH of the sediments went from 7.15 to 6.87 suggesting an overall acidification of the sediments. Other sediment quality parameters (ash free dry weight and water content) were stable over the production period. Chlorophyll concentration was highly variable.

Plasmid pSFn1 PCR specificity and PCR detection limits

Eleven species of *Vibrio* and 34 strains of *Vibrio nigripulchritudo* were used to check the specificity of the primers designed. No amplification was generated when using template DNA from strains belonging to a different species than *V. nigripulchritudo*. DNA from *V. nigripulchritudo* strains known to carry the plasmid pSFn1 (Reynaud et al., 2008) systematically generated an amplicon at the expected size, 954 bp (Table II, SFn1 group, n = 6.) Other strains with the summer syndrome genotype and known to be highly pathogenic also generated a 954-bp product with the plasmid specific PCR (Table II, Fn13 group, n = 4). DNA from strains described as moderately pathogenic did not generate any amplicon (Table II, BDn1 group, n = 8). All the strains with a different genotype than the summer syndrome genotype were negative with the pSFn1 specific PCR, whether moderately or non virulent (Table II, SO65 group, n = 5 and SFn118 group, n = 11). Virulent strains with the summer syndrome genotype can now be distinguished as pSFn1-carriers or non-carriers. The technique was then optimized for the analysis of sediment samples.

It was shown previously that the TCBS medium specific of *Vibrio* is not suitable for the detection of *V. nigripulchritudo* because this species grows poorly on this medium. Therefore, Marine agar supplemented with 2% glycerol is used, but this medium is not selective. For *V. nigripulchritudo* colonies to be detected on this medium from sediment samples, they need to be sufficiently concentrated and not overshadowed by the other colonies. Our results indicate

that the detection limit for presumptive *V. nigripulchritudo* on Marine Agar is 10^3 CFU.g^{-1} wet sediment weight.

Using a *V. nigripulchritudo* species specific PCR on crude sediments inoculated with decreasing quantities of *V. nigripulchritudo*, the detection limit was also found to be 10^3 CFU.g^{-1} wet weight. The same samples were used to evaluate the detection limit of the pSFn1 specific PCR that was about 10^6 CFU.g^{-1} wet sediment weight.

Using DNA from enriched sediments samples allowed lowering the detection limits to about 100 CFU.g^{-1} wet weight for both *V. nigripulchritudo* species specific and pSFn1 virulence marker PCRs.

Sediment total heterotrophic flora (THF)

Between both sampling dates, cultivable THF is comparable ($5.13 \cdot 10^7$ CFU.g^{-1} and $3.24 \cdot 10^7$ CFU. g^{-1}) At the first sampling date, no spatial variability is evidenced in terms of THF (figure 1). At the second sampling date, THF is slightly higher in the center of the pond and towards the lower part near the water exit station.

***Vibrio nigripulchritudo* detection in sediment at different genetic levels**

At the species level, the number of sediment sample cultures presenting black colonies was 26/42 at D56 before the outbreak, and 37/42 at D107 after the mortality outbreak (days 89-95, figure 1). Direct PCR detection of the species went from 0/42 to 30/42 positive sediment samples (figure 2). Analysis of sediments after enrichment reveals that all samples are positive at day 56 and 107 (figure 3).

At the emerging "Summer Syndrome" cluster level, the sediment samples were screened for this cluster by real time PCR using the method described previously (Goarant et al., 2007). Using the standard curve established previously, the detection limit for this assay is 100 inoculated CFU.g^{-1} wet weight. Before the outbreak (D56), only one sample proved positive for this cluster whereas 36/42 points were found positive after the outbreak (D107) (figure 2). Samples with the highest concentration of *V. nigripulchritudo* with the "Summer Syndrome" genotype ($> 4.10^3$ CFU.g^{-1} wet weight) do not follow a particular spatial pattern in the pond (figure 4). Highly concentrated samples (as represented by black squares in figure 4) are spread over the entire pond area. Some samples are positive with the species PCR and negative for the pathogenic cluster. More surprisingly, some samples are positive for the pathogenic cluster but are negative with the species PCR.

At the plasmid level with direct PCR, only one sample was positive at D56 and none at D107 (figure 2). To lower the detection limit with this PCR technique (10^6 cfu.g^{-1} wet weight), an enrichment step was added before DNA purification from sediments. Heterotrophic bacteria carrying pSFn1 and present at low concentrations in the sediments can be detected by PCR after enrichment, subject to the fact that the bacteria are cultivable in the enrichment medium. With PCR on enriched sediments, all the samples were positive at D56, but at D107, only 29/42 points were positive (figure 3).

4. Discussion

To understand how the environment can influence pathogen development in ponds, several parameters were measured on 42 samples of an experimental grow out pond. A "Summer

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Syndrome" mortality outbreak caused by HP *V. nigripulchritudo* occurred and one sampling survey could be conducted both before and after this outbreak. At both dates, we used both culture-dependant and molecular techniques to characterize *V. nigripulchritudo* strains in the sediments in order to circumvent the limits of each technique: lack of specificity for culture-dependant techniques, high detection limit for direct PCR, lack of quantification for enriched PCR. Specific PCR assays were used to identify the etiological pathogen at different genetic levels. Techniques described previously allowed the identification of *V. nigripulchritudo* at the species level and at the level of the cluster that contains all Summer Syndrome HP isolates (Goarant et al., 2006b and 2007). Lastly, a plasmid PCR assay targeting pSFn1 developed here was used both to study pathogen dynamics in the farming environment during production and to confirm the identification of the etiological agent of the outbreak. Because plasmids are mobile genetic elements, we checked and validated that our assay failed to amplify any product from non-*V. nigripulchritudo* strains or from *V. nigripulchritudo* strains with no or only moderate virulence to shrimp. Though not isolated, some strains could carry the plasmid but not the chromosomic background necessary for virulence expression. Therefore, it is useful to analyze both the genotype (based on a chromosomal gene polymorphism) and the presence of pSFn1 in newly isolated strains and check that they belong to the Summer Syndrome emerging genotype. Because plasmids may be lost in subcultures, we also checked the stability of pSFn1 which could be identified in cultures after more than 15 passages (our unpublished results).

When analyzing the results from all the techniques used together in this study, a picture of *V. nigripulchritudo* strains dynamics in sediments at two phases of the production cycle can be suggested.

Total Heterotrophic cultivable Flora was shown to be more concentrated in the center of the pond, especially at the end of the rearing period (figure 1). This is consistent with the results of Burford et al (1998) and the ones of Delgado et al (2003) on ponds equipped with aerators showing that THF was significantly more concentrated in the pond center, where sludge had accumulated. Delgado and coll. also demonstrated a pH decrease as observed in our study ; Burford and coll. also showed that bacterial numbers were correlated with organic carbon and nitrogen content. Similarly, aquaculture-influenced sediments were shown to harbor high levels of *Vibrio* compared to surrounding areas in other systems, either fish (Enger et al., 1989) or mollusks (La Rosa et al., 2001). Culture on Petri dishes showed that putative *V. nigripulchritudo* colonies were present at the first sampling date and spread around the outskirts of the pond. This fact is interesting since this area was shallower and the environmental conditions could be different in terms of light exposure, oxygen content, and nutrient concentration, leading to a different colonization dynamics by benthic organisms. *Vibrio* are known to have an affinity for phytoplankton and it was shown that *Vibrio cholerae* developed in environments favorable to algae, cyanobacteria, and aquatic plants (Pruzzo et al., 2005). Similar results were obtained by Eiler and colleagues (2007) who demonstrated that cyanobacterial by-products largely influenced *Vibrio* population growth in mesocosms. Early studies evidenced this trophic association and pointed out its interest as an explanation to pathogen persistence (Tamplin et al., 1990 ; Epstein et al., 1993 ; Islam et al., 1994). This particular affinity between *Vibrios* and planktonic food chains was also studied in more details in recent large scale studies (Lipp et al., 2002), including environmental sensing for epidemics prediction (Hsieh et al., 2008 ; Constantin de Magny et al., 2008).

Theoretically, the detection limits for putative *V. nigripulchritudo* colonies on Petri dishes and for identification at the species level with direct PCR are both around 10^3 CFU.g^{-1} wet sediment weight. However, in use, culture-dependant detection seems to be more sensitive but has major drawbacks: cells need to be in a cultivable state, presumptive phenotypic identification based on the sole expression of a pigment can suffer a lack of specificity, plates tend to be overcrowded by the THF and black colonies are difficult to isolate. Lastly, because *V. nigripulchritudo* contains both saprophytic and pathogenic strains, culture-dependant detection leaves some uncertainty.

At the first sampling date, D56 of the grow out period, a population of pSFn1-carrying bacteria as well as bacteria belonging to the species *V. nigripulchritudo* are evidenced over

the entire pond sediments by PCRs from enriched sediment samples. These bacteria were not easily detected by direct PCRs that only detect populations above 10^3 CFU.g^{-1} (for the species) or 10^6 CFU.g^{-1} (for the plasmid), demonstrating their relatively low level. One sample was nevertheless positive for the plasmid, and another one was positive for the emerging genotype, possibly indicating that some sediment areas support high concentrations of HP *V. nigripulchritudo*, even before the mortality event. Sediments could therefore actually be the source of infection, as hypothesized by Goarant et al (2006a).

Why these sediment samples were not positive with the species-specific direct PCR remains unknown: this could be a technical artifact due to heterogeneity in DNA extraction or difference in PCR efficiencies. Alternatively and though pSFn1 was never evidenced from any other bacterial species, pSFn1 or a highly similar plasmid could possibly be present in non-*V. nigripulchritudo* bacteria in our experimental pond.

At the second sampling date, D107 of the production cycle, which is close to the end of the 112-day rearing period, the sediments were colonized by *V. nigripulchritudo*, including strains with a genotype identical to that of the emerging cluster, as evidenced by the results obtained with direct PCR (figure 2). This shows that over time, the proportion of bacteria from the species *V. nigripulchritudo* including those of the emerging genotype increased. Between the two sampling dates and possibly related to the mortality event, the sediments were colonized by the *V. nigripulchritudo* strains, reaching concentrations above the detection limit of direct PCR. Again, some samples proved positive for the emerging genotype but not for the species, most probably because of the differences in detection limits between classic PCR used for species characterization and real time PCR used for genotype quantification (a one-log factor).

Surprisingly, bacteria harbouring the plasmid pSFn1 were less frequently found in the sediments at this sampling date, after the mortality event, even though both the species *V. nigripulchritudo* and the pathogenic cluster were shown to colonize the sediments. Culture-based evaluation of *V. nigripulchritudo* populations confirms that this species tends to colonize the sediments during the production cycle.

Despite the overall colonization of the sediments by strains of *V. nigripulchritudo*, and the very high production of pSFn1-harbouring strains during the mortality event, the detection of pSFn1-carrying bacteria was not increased at the second sampling date. Furthermore, plasmid detection with PCR after sample enrichment was more difficult than at the first sampling date. This is unexpected since the species and emerging genotype were both detected at higher concentrations at the second sampling date with either direct or enriched PCR. Though a bias can possibly result from enrichment procedures, enriched PCR results suggest that highly pathogenic strains carrying the plasmid are not as frequent at D107 than at D56. We hypothesize that this plasmid could constitute a metabolic burden in the late pond environment. In contrast, highly virulent strains carrying pSFn1 are constantly isolated from moribund shrimp during Summer Syndrome outbreaks, suggesting that this plasmid confers a selective advantage in diseased shrimp and is actually related to virulence, as suggested by Reynaud et al. (2008). We make the hypothesis that the selection of highly pathogenic strains takes two steps: first, *V. nigripulchritudo* from the Summer Syndrome emerging genotype are selected in the eutrophic pond environment and second a subpopulation of cells from this genotype carrying the plasmid are selected in weakened shrimp as another ecological niche. Should this hypothesis be later confirmed, this would illustrate how genomic diversity and horizontal gene transfer allow the selection of the fittest strains in any specific environment even though that environment might be changing and new ecological niches might appear (Hacker et al., 2004).

Since there are no means to eliminate bacterial pathogens directly in ponds, knowledge on pathogenic strain dynamics during grow-out and dry-out periods could lead to methods to limit pathogen development in sediments. Because of the high diversity in virulence capacity of *V. nigripulchritudo* among New Caledonian isolates, molecular tools targeting the etiological agent of Summer Syndrome had to be improved. Highly pathogenic (HP) *V. nigripulchritudo* all belong to the same emergent cluster. However, this cluster contains both HP strains and moderately pathogenic (MP) strains (Goarant et al., 2006b) and HP strains can only be distinguished from MP strains by the presence of the pSFn1 plasmid, regarded as a virulence marker (Reynaud et al., 2008). The PCR assay targeting this plasmid developed here allows, together with a genotype-specific PCR assay, to evaluate the risk of Summer Syndrome in New Caledonian shrimp aquaculture. Further studies will study the occurrence of *V. nigripulchritudo* populations after the harvest and during the dry out period.

Acknowledgements

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Tables

Table I. Reference strains used in this study

Species	Strain
Vibrio nigripulchritudo	CIP 103195[T]
Vibrio penaeicida	KH-1[T] (=IFO 15660[T])
Vibrio penaeicida	CRB AM101
Vibrio orientalis	LMG 7.897
Vibrio nereis	LMG 3.895
Vibrio splendidus	CIP 102.893
Vibrio alginolyticus	ATCC 17.749[T]
Vibrio harveyi	CIP 103192[T]
Vibrio fluvialis	CIP 103.355[T]
Vibrio aesturianus	CIP 102971[T]
Vibrio damsela	CIP 102.761[T]
Vibrio orientalis	LMG 7.897

CIP = Collection de l'Institut Pasteur, Paris, France; IFO = Institute for Fermentation, Osaka, Japan ; CRB = Centre de Ressources Biologiques, Laboratoire Génétique et Pathologie, Ifremer, La Tremblade, France ; LMG = Belgian Coordinated Collection of Microorganisms, Laboratory voor Microbiologie, Universiteit, Gent, Belgium.

Table II. *Vibrio nigripulchritudo* field isolates used and recent results on their virulence and genetic characterization.

Vibrio nigripulchritu do strains	SFn1 group : SFn1, AgMn7, SFn27, SFn135, POn3, POn19.	Fn13 group : Fn13, SVn7, SVn8, SVn9.	BDn1 group : BDn1, BLFn1, BLFn2, ENn2, AQn1, AQn2, Wn1, Wn3.	SO65 group : SO65, AM102, AM115, AMn3, AMn4.	SFn118 group : SFn118, POn4, POn13, SOn2, AgMn1, AgMn3, ESn2, Fn2, POn12, ENn1, SOn1.
Virulence in experimental infection	Highly pathogenic (Goarant et al., 2006a and 2006b and Reynaud et al., 2008)	Highly pathogenic (this study)	Moderately pathogenic (Goarant et al., 2006a and 2006b and Reynaud et al., 2008)	Moderately pathogenic (Goarant et al., 2006b and unpublished results)	Non virulent (Goarant et al., 2006a and 2006b)
Genotypic characterization (Goarant et al., 2006b)	Summer syndrome genotype	Summer syndrome genotype	Summer syndrome genotype	Different genotype	Several different genotypes
Plasmid analysis and macroarray results (Reynaud et al, 2008)	All pSFn1 markers detected by macro-array	No data available	Some pSFn1 markers not detected by macro-array	No data available	No plasmid markers
PCR results from this study using pSFn1-specific primers pSFn1-6020 and pSFn1-6974	Positive	Positive	Negative	Negative	Negative
Suggested diagnosis in terms of Summer syndrome hazard	High risk	High risk	Medium risk	Medium risk	Low risk

Table III. Sediment quality. **Mean** (standard error; sample size) of samples collected over the entire pond.

Sampling date	Day 56	Day 107
Temperature (°C)	**23.8** (0.1; 42)	**29.7** (0.1; 42)
pH	**7.2** (0.1; 42)	**6.9** (0.1; 42)
Chlorophyll Concentration ($\mu g.g^{-1}$ dry weight)	**26** (4; 41)	**6** (1; 42)
Organic matter (%)	**4.5** (0.3; 37)	**4.9** (0.2; 40)
Water content (%)	**25.6** (0.6; 42)	**25.1** (0.7; 42)
Total heterotrophic flora (CFU. g^{-1} dry weight)	**$5x10^7$** ($5x10^6$; 84)	**$3x10^7$** ($3x10^6$; 62)

Figure 1. Culturable total heteretrophic bacteria (log CFU.g^{-1} dry weight) of pond samples at two sampling dates. Circles represent samples from which presumptive *Vibrio nigripulchritudo* were detected. Darker areas represent higher bacterial counts. Pond dimensions are in meters.
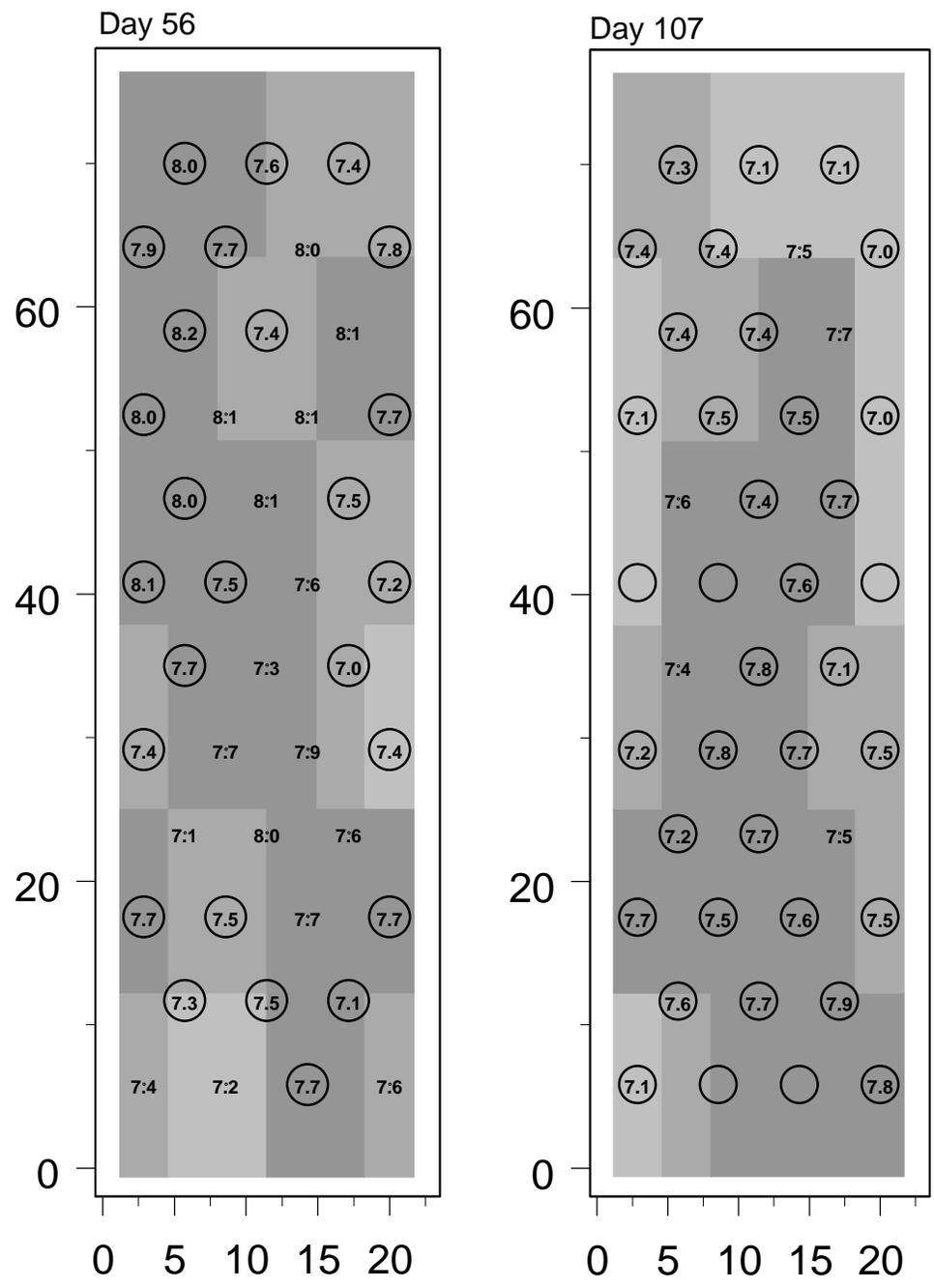
Figure 2. Direct PCR detection of *Vibrio nigripulchritudo* (circles), Summer Syndrome genotype (squares) and plasmid pSFn1 (crosses) at two sampling dates. Summer Syndrome genotype detection was quantitative and four classes are identified by squares of four sizes and shades of grey (> 4×10^3, $2 \times 10^3 - 4 \times 10^3$, $<2 \times 10^3$ *Vibrio*.g^{-1} wet weight, not detected.) Pond dimensions are in meters.
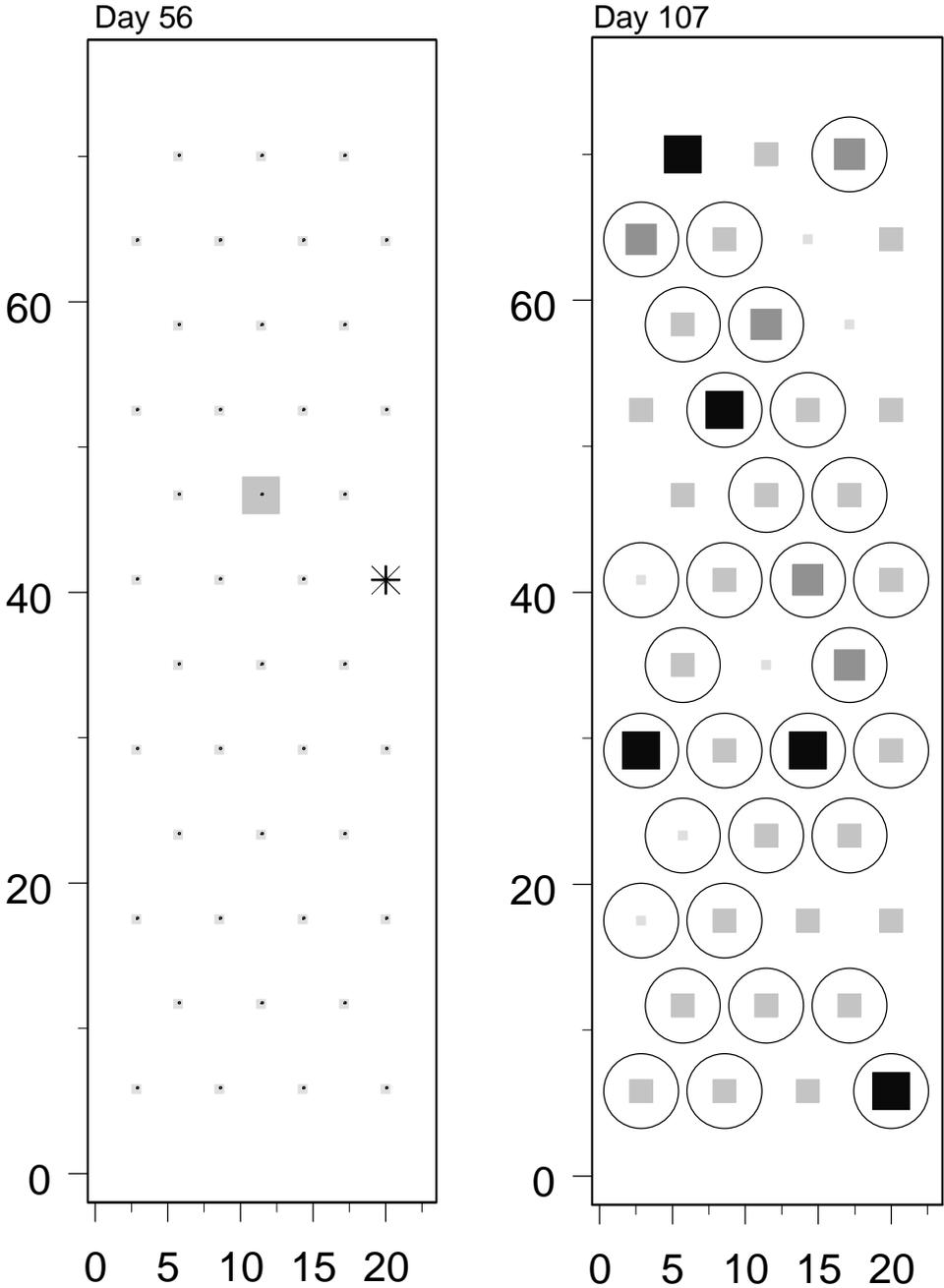
Figure 3. Enriched PCR detection of *Vibrio nigripulchritudo* (circles) and plasmid pSFn1 (crosses) at two sampling dates. Pond dimensions are in meters.