"Summer Syndrome" in Litopenaeus stylirostris in New Caledonia: Pathology and epidemiology of the etiological agent, Vibrio nigripulchritudo

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Abstract: The Summer Syndrome is a new shrimp disease that has been affecting a shrimp growout farm in New Caledonia since end of 1997. It was recognized to be caused by a systemic vibriosis due to Vibrio nigripulchritudo. This new disease turned out almost immediately enzootic in the shrimp farm involved and has affected all its crops ever since. Since the year 2000, V. nigripulchritudo strains have been found in several shrimp farms, although Summer Syndrome is still limited to one particular area, affecting, since 2003, two adjoining farms. As part of a multidisciplinary research program, a high-frequency survey was carried out during the summer 2002–2003 in two shrimp farms: one affected farm and another one in which pathogenic strains could be isolated but without any disease event. It permitted a good description of V. nigripulchritudo dynamics in shrimp and the ecosystem in both farms. The study of virulence characteristics of some isolates showed that pathogenic and non-pathogenic strains occur in the shrimp farms environment and that both may be found at the same time in one farm. Our results strongly suggest a persistence of pathogenic strains in wet zones of the pond sediment at low concentrations between crops in the affected farm, and their development during the rearing cycle as a cause of shrimp infection.

Keywords: Shrimp; Diseases and their control; Vibrio; Epidemiology

Introduction

Vibriosis is a major disease problem in almost all farmed marine animals, including penaeid shrimp (Lightner and Lewis, 1975; Takahashi et al., 1985; Lightner, 1988; Brock and Lea Master, 1992; Mohney and Lightner, 1994). However, it has only received poor research attention so very poor knowledge has been gained on this pathology. This is due, in part, to the authority given to treat with antibiotics in many producing countries. Nevertheless, the massive use of antibiotics has led to the selection of antibiotic-resistant bacterial strains (Brown, 1989; Holmström *et al.*, 2003) and the inefficacy of such an approach. Furthermore, some producing countries, including New Caledonia, have totally banned the use of antibiotics in growout ponds and most importing countries have decreased tolerance on antimicrobial residues. Therefore, research programs on shrimp vibriosis are now being developed. New Caledonia is a small island country in the South Pacific and its shrimp industry benefits from an almost virus-free status, IHHNV being the only significant virus present, and its domestic stock of *Litopenaeus stylirostris* being resistant to it (Weppe *et al.*, 1992). Still, from 1993 on, the shrimp growers had to face a seasonal vibriosis which was named Syndrome 93 (Goarant *et al.*, 1996 and 1999; Mermoud *et al.*, 1998). First observed in December 1997, a new vibriosis, which was named "Summer Syndrome", is causing increasing worries about the profitability of the industry. Therefore, the shrimp farmers' association is placing the research on vibriosis as the number one R&D priority. As a response, the Aquaculture Department of Ifremer (the French Institute for Marine Sciences) has developed a specific research program, named DeSanS (possibly translated as *Stylirostris* Health Challenge). This research program is based on a multidisciplinary approach associating rearing technology, pond ecosystem studies, shrimp ecophysiology and immunology, nutrition, pathology and genetics. It aims at gaining an overall explanation scheme of the seasonal vibriosis (Harache and Herbland, 2004) as a contribution to strengthen the sustainability of New Caledonian aquaculture.

A brief history of the "Summer Syndrome"

Vibrio nigripulchritudo was first isolated in New Caledonia in 1995 from diseased shrimp affected by the winter vibriosis Syndrome 93 (Costa *et al.*, 1998). It was at this time restricted to two adjoining farms and was considered as a geographically restricted phenomenon (Goarant *et al.*, 1999). Zoosanitary recommendations were given in order to minimize the risk of spreading of these pathogenic strains. However, *V. nigripulchritudo* was again isolated in late December 1997 in moribund shrimp from two ponds in one farm located 50 kilometres southern from the original isolation locations. It was also causing a septicemic vibriosis, but in high water temperature conditions that did not fit the Syndrome 93 epidemiology, which typically occurs when water temperature is below 25°C (Mermoud *et al.*, 1998 ; Goarant *et al.*, 2000). The histological findings indicated an acute systemic vibriosis (D.V. Lightner, com. pers.; J. Brock, com. pers. ; O. Ferré, com. pers.). Experimental infections using moribund shrimp carcasses ultrafiltrates failed to induce any mortality in healthy shrimp. This new disease was therefore considered as an acute systemic vibriosis caused by *Vibrio nigripulchritudo*. In the affected farm, the disease turned out almost immediately enzootic, with a very stereotyped epidemiology, taking place after 50 to 60 days rearing in every summer crop and causing high mortalities. Healthy carriers were frequently observed in the affected farm, both before, during and after the outbreaks (Goarant *et al.*, 2004). *V. nigripulchritudo* was also isolated from both healthy and moribund shrimp in many other farms, in which it was not responsible for any significant mortality, or only caused low mortality with typical opportunistic features: affecting only a few molting animals or its expression could be related to inadequate pond conditions and stopped as soon as the conditions returned to normal. In the Summer Syndrome affected farm, strains of *V. nigripulchritudo* could also be isolated from pond water, sediment pore water, renewal water or associated with many Crustaceans both in the ponds and in the pumping water, and in zooplankton samples from the ponds (Goarant *et al.*, 2004). The isolated strains demonstrated different virulence patterns in experimental infection with healthy

shrimp. As part of the DeSanS program, a wide field survey was carried out during the 2002-2003 summer season based on a multidisciplinary approach to the Summer Syndrome (Harache and Herbland, 2004).

Material and methods

Survey and growout ponds

A survey was carried out in two 3-hectare earthen ponds: one pond in a farm that is affected by the Summer Syndrome (farm "DF" for Diseased Farm) and another pond in a farm in which the Summer Syndrome is not observed, though pathogenic *V. nigripulchritudo* strains have already been isolated (farm "HC" for Healthy Control) (Goarant *et al*., 2004). Both ponds were stocked on the same day with postlarvae (PL) originating from the same hatchery batch, with 28 PL.m^{-2} in farm DF and 35 PL.m^{-2} in farm HC. Both ponds were equipped with aerators. Shrimp were fed the same commercial feed pellet and managed by the technical staff of each own farm according to their usual techniques. After being drained from the previous crop, pond bottoms were ploughed and maintained as dry as possible, by pumping water in puddles. The ponds were filled ca. 2 weeks before seeding the postlarvae, according to each own farm's usual technique. The rearing period began in October 2002 and lasted until March 2003.

Shrimp and mortality study

Shrimp sampled with a castnet were considered healthy if displaying no lethargy or abnormal behaviour. Moribund shrimp were manually caught on the pond edges. Pond daily mortality was evaluated by counting dead and moribund shrimp on pond edges twice a day at 6.00 AM and 3.00 PM. Actually, an accurate estimation of daily mortality in a 3-ha shrimp pond is almost impossible, due to the wide area, high turbidity and cannibalism. Therefore, only a minor part of dead animals can be observed. Overall survival rates were calculated as the ratio of live shrimp harvested at the end of the growout period to the number of postlarvae initially seeded in the ponds.

Sampling and analysis

On the day of stocking, 100 postlarvae were randomly taken, rinsed with sterile seawater, crushed in an equal volume of sterile seawater and used for bacteriological analysis. Thirty healthy individual juvenile animals were then sampled on days 25 and 26 in farms DF and HC respectively. From day 32 on, 30 healthy individual juveniles were sampled on even dates in farm DF and on uneven dates in farm HC. Moribund shrimp were also sampled over the mortality period in order to confirm the aetiology of the outbreak. The haemolymph of juvenile shrimp (healthy or moribund) was withdrawn from the ventral sinus after rinsing with sterile seawater and used for bacteriological analysis. Pond water, sediment and renewal water were sampled at 6.00 AM, 3 and 2 days before stocking on farms DF and HC respectively, then on even dates in farm DF and on uneven dates in farm HC for renewal and pond water, and every 7 days for the sediment pore water. Pond water was hundred-fold diluted in sterile seawater. Sediment pore water was extracted by low-speed centrifugation (200 g for 5 minutes) and ten-fold diluted. Renewal water was ten-fold diluted. In any case, 100 µL of all these diluted samples were used for bacteriological analysis in triplicate. All samples (crushed postlarvae, diluted water samples, heamolymph) were plated on Marine Agar with 2% (w/v) glycerol added, on which *V. nigripulchritudo* colonies produce a dark grey to black pigment (Baumann and Schubert, 1984). Petri dishes were incubated for 72 hours at 30°C.

Vibrio nigripulchritudo *identification scheme*

Colonies displaying a dark grey to black coloration were considered as presumptive *V. nigripulchritudo*. Some were isolated and the identification was confirmed by classical phenotypical tests: Gram, morphology, motility, oxydase and an API 20E strip (Bio Mérieux, Marcy l'Etoile, France). Lastly, if these tests were in agreement with the identification as *V. nigripulchritudo*, a PCR confirmation was obtained with the specific primers VnF (5'-GTGTGAATTTAATAGATGCACATT-3' and VR (5'-CGCATCTGAGTGTCAGTATCT-3') with an annealing of 30 seconds at 58°C (Saulnier, unpublished data). The specificity of this PCR was validated over 18 *Vibrio* species, including the major shrimp pathogens (*V. penaeicida*, *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*). The isolates were then preserved in 16% glycerol at -80°C.

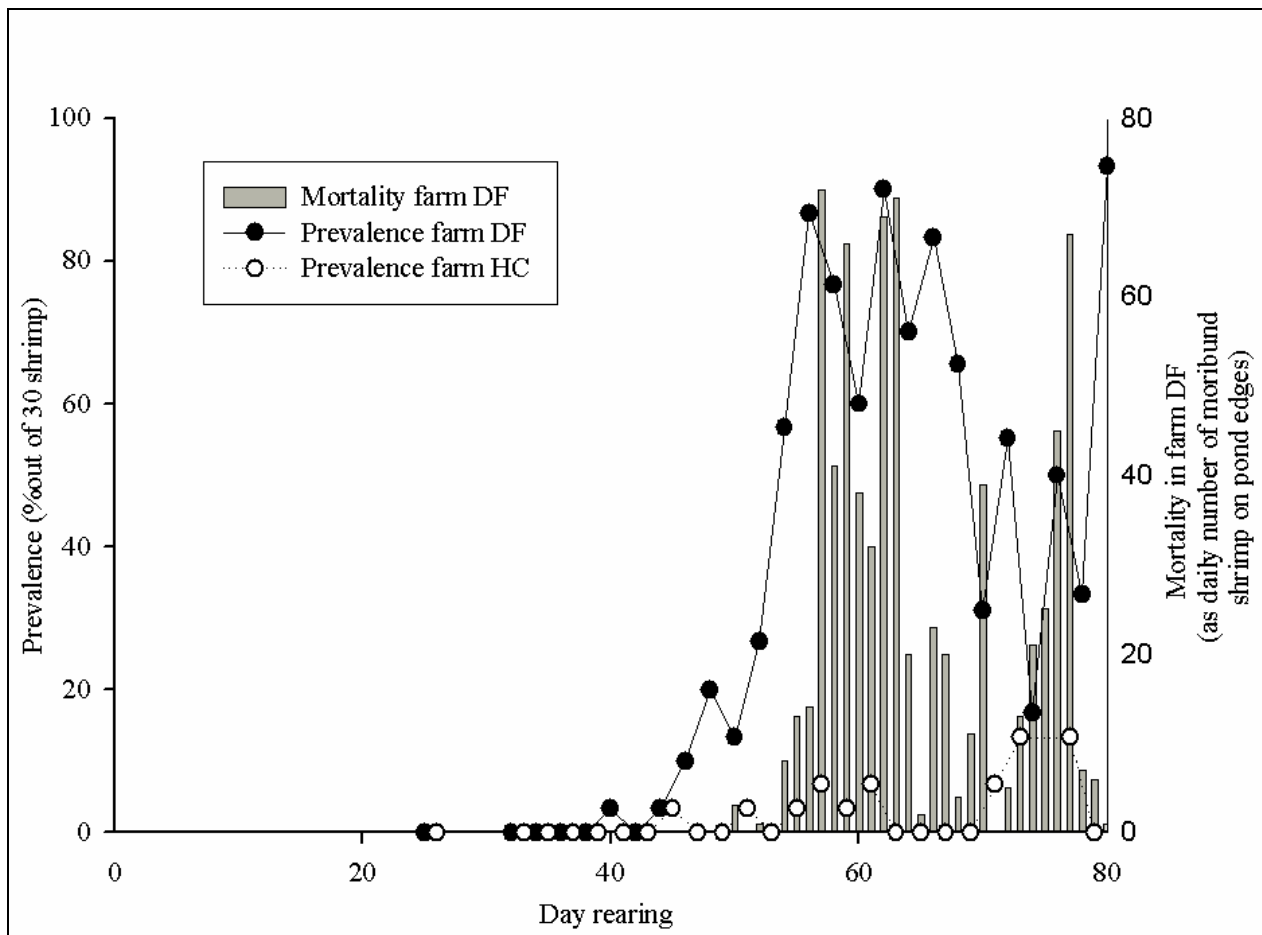### *Strain virulence classification*

The *V. nigripulchritudo* strains were cultured in Marine Broth for 18 hours at 30°C under constant shaking, allowing to reach the late exponential growth phase. The bacterial culture concentrations were evaluated by reading their optical density at 600 nm, as compared to a reference curve previously determined (data not shown). Fifty to 500 colony forming units were injected intramuscularly in healthy juvenile *Litopenaeus stylirostris* kept in aquaria (2 replicates aquaria with 8 to 10 shrimp in each for each strain) filled with filtered seawater, aerated and held at 27°C. Additionally, higher concentrations of a non-virulent isolate were also injected to evaluate an effect of infective dose on this classification scheme. Control shrimp were injected an equal volume of sterile culture medium. Survival was followed over a 3-day period, because preliminary trials demonstrated that no significant mortality occurred after this duration. The experiment was validated only if all control shrimp survived. Most of the strains tested were isolated from farms studied during the survey, but additionally, strains from different origins were tested, in order to gain a better understanding of the epidemiology of *V. nigripulchritudo* in New Caledonian shrimp aquaculture.

Results and discussion

Validity of the comparison:

Though simple, the specificity of the method used for the counting of *V. nigripulchritudo* in the pond ecosystem and the haemolymph of both healthy and diseased shrimp was validated by phenotypic and PCR confirmation of 39 out of 43 strains (91%) isolated during the survey. From this sampling, it can be assumed that *V. nigripulchritudo* counts during the survey have a reasonable specificity. Some mortality was observed, in farm DF only, after 50 days rearing (figure 1). In contrast, no mortality related to *V. nigripulchritudo* was observed in farm HC. The mortality in farm DF was confirmed to be typical "Summer Syndrome" by evidencing a septicemia caused by *V. nigripulchritudo* in 87 moribund shrimp out of 92 sampled at different time-points of the mortality peak between days 50 and 82. This confirms that the pond studied was affected by the same disease than previously observed and named Summer Syndrome. The overall survival rates were 27% and 62% in farms DF and HC respectively, which are also typical of the average survivals obtained in both farms during the last few years.

Figure 1: *Vibrio nigripulchritudo* observed prevalence in shrimp from both farms and Summer Syndrome mortality in farm DF. Mortality is expressed as "daily number of moribund shrimp observed on pond edges" as explained in the text.

Strain virulence classification

Sixty nine *V. nigripulchritudo* strains, both clinical and environmental isolates, were tested by experimental infection. Cumulative mortality 72 hours after infection ranged from 0 to 100%. The strains were considered as pathogenic if mortality was higher than 20% and non-pathogenic if mortality was under 20%. A non-virulent isolate (SFn118) proved to be non-pathogenic even when injecting $7.5x10^4$ CFU.shrimp^{-1}. These results are summarized in table 1. Current work is in progress to identify virulence genes in pathogenic *V. nigripulchritudo* isolates in order to permit a better distinction between pathogenic and non-pathogenic *V. nigripulchritudo* strains and to conduct a more accurate epidemiological description using virulence-based diagnostic tools (Gay *et al*., 2004).

Table 1: Strain virulence classification of 69 *Vibrio nigripulchritudo* field isolates, indicating the isolation source, dose used for infection and survival after 72 hours. Experiments were conducted as described in Material and methods section.
Underlined strains were isolated during the survey (the rearing day is indicated for these strains).
Bold strains were isolated from farms affected by the Summer Syndrome.
Italics strains were isolated from Syndrome 93 (cool season) mortality episodes.

strain	Farm	Source	Day rearing (survey) or isolation date	CFU.shrimp^{-1}	survival (%)
<u>SFn127</u>	DF	healthy shrimp	d40	383	0
<u>SFn128</u>	DF	moribund shrimp	d54	319	0
<u>SFn142</u>	DF	renewal water	d54	925	8
<u>SFn135</u>	DF	pond water	d55	774	0
<u>SFn137</u>	DF	pond water	d55	396	17
<u>SFn144</u>	DF	Sediment	d60	514	100
<u>POn 8</u>	HC	healthy shrimp	d45	116	83
<u>POn 9</u>	HC	healthy shrimp	d51	94	100
<u>POn 12</u>	HC	healthy shrimp	d71	53	100
<u>POn 13</u>	HC	healthy shrimp	d73	68	100
<u>POn 14</u>	HC	healthy shrimp	d73	40	83
<u>POn 15</u>	HC	healthy shrimp	d73	47	100
<u>POn 16</u>	HC	healthy shrimp	d71	556	100
<u>POn 17</u>	HC	Sediment	d73	700	100
<u>POn 18</u>	HC	healthy shrimp	d77	294	100
<u>POn 19</u>	HC	healthy shrimp	d77	700	0
SFn1	DF	moribund shrimp	March 2000	17,5	0
SFn2	DF	moribund shrimp	March 2000	630	0
SFn27	DF	sédiment	December 2000	36	0
SFn32	DF	healthy shrimp	December 2000	500	60
SFn48	DF	moribund shrimp	November 2001	124	6
SFn49	DF	pond water	November 2001	94	0
SFn106	DF	moribund shrimp	February 2002	44,5	0
SFn111	DF	crab shell	March 2002	197	88
SFn115	DF	renewal water	March 2002	255	100
SFn117	DF	renewal water	April 2002	254	100
SFn118	DF	renewal water	April 2002	75	100
				750	92
				7500	100
				75000	100
SFn119	DF	moribund shrimp	October 2002	114	100
POn2	HC	healthy shrimp	April 2002	253	0
POn3	HC	healthy shrimp	April 2002	60	0
POn4	HC	healthy shrimp	April 2002	397	100
AgMn1	affected	healthy shrimp	October 2003	287	94
AgMn2	affected	healthy shrimp	October 2003	55	88
AgMn3	affected	healthy shrimp	October 2003	64	100
AgMn4	affected	healthy shrimp	November 2003	215	100
AgMn5	affected	healthy shrimp	November 2003	147	100
AgMn6	affected	healthy shrimp	November 2003	42	100
AgMn7	affected	healthy shrimp	November 2003	120	0
AgMn8	affected	moribund shrimp	November 2003	16	0
AgMn9	affected	pond water	November 2003	15	0
AgMn10	affected	moribund shrimp	November 2003	98	0
AgMn12	affected	Sediment	November 2003	10	0
AgMn13	affected	Sediment	November 2003	34	0
AMn3	not affected	moribund shrimp	March 2003	145	6
AMn4	not affected	moribund shrimp	March 2003	121	0

AMn5	not affected	moribund shrimp	March 2003	220	0
AQn1	not affected	healthy shrimp	March 2003	108	50
AQn2	not affected	healthy shrimp	March 2003	220	19
BDn1	not affected	healthy shrimp	February 2003	124	19
BDn2	not affected	healthy shrimp	February 2003	206	31
BLFn1	not affected	moribund shrimp	March 2001	530	27
BLFn2	not affected	moribund shrimp	December 2001	266	25
BLFn4	not affected	moribund shrimp	December 2001	383	31
ENn3	not affected	healthy shrimp	June 2000	600	100
ESn1	not affected	healthy shrimp	June 2000	320	100
ESn2	not affected	healthy shrimp	June 2000	200	100
ESn3	not affected	healthy shrimp	June 2000	450	92
Fn2	not affected	healthy shrimp	February 2003	112	94
FTn1	not affected	moribund shrimp	January 2001	233	88
FTn2	not affected	healthy shrimp	March 2003	86	38
MT 3	not affected	healthy shrimp	June 2000	420	100
SBn2	not affected	healthy shrimp	March 2003	148	38
SVn3	not affected	healthy shrimp	February 2003	195	100
Wn1	not affected	moribund shrimp	January 2001	46	56
Wn13	not affected	moribund shrimp	January 2001	106	50
Wn14	not affected	moribund shrimp	November 2002	89	50
Wn3	not affected	moribund shrimp	November 2002	60	50
SO65	*not affected*	*moribund shrimp*	*May 1995*	*20*	*0*
AM115	*not affected*	*moribund shrimp*	*May 1995*	*146*	*12*

Animals

V. nigripulchritudo could not be detected in the postlarvae seeded in the ponds. Furthermore, it has not been detected from other postlarvae batches during preliminary studies (unpublished data). Therefore, it can be considered that the postlarvae are *V. nigripulchritudo*–free at the time of stocking and that the animals get infected while in the growout ponds.

In farm HC, *V. nigripulchritudo* was first detected on day 45 in 1 shrimp out of 30. It was then occasionally found, but in no more than 4 shrimp out of 30 (figure 1). Nine presumptive *V. nigripulchritudo* strains were isolated from healthy shrimp between days 45 and 77 and all confirmed to be *V. nigripulchritudo*. All but one tested as non-virulent when injected to healthy *L. stylirostris*. The only pathogenic strain was isolated from a presumed healthy shrimp on day 77, a few days before the end of the survey. Though isolated late in the growout period, this result demonstrates that highly pathogenic strains were also present in farm HC.

In farm DF, *V. nigripulchritudo* was first detected in shrimp (1 out of 30) on day 40 and the isolated strain proved to be virulent (see table 1). From day 46 on, the prevalence increased and then fluctuated between 5 and 28 shrimp out of 30 until the end of the survey (figure 1). The first moribund shrimp was observed on day 50, which is very typical of the Summer Syndrome. Interestingly, the increase in the mortality curve from day 50 to day 60 is almost parallel to the increase in prevalence from day 40 to day 50, suggesting that the disease has a latency phase of ca. 10 days. Pathogenic strains could be isolated from apparently healthy animals. Furthermore, after the first mortality peak, the prevalence fluctuates (figure 1). The overall survival in farm DF (27%) also demonstrates that the prevalence can be higher (up to 28 out of 30) than the mortality. Taken together, these results indicate that *V. nigripulchritudo* is possibly cleared from the haemolymph and that shrimp that carry *V. nigripulchritudo* may remain healthy or even recover, as experimentally demonstrated with other *Vibrio* species (Alday-Sanz *et al.*, 2002; Van-De-Braak *et al.*, 2002). Different experimental infection modes are currently being studied as to investigate the course of infection (Saulnier *et al.*, 2000) and possible recovery.

Pond ecosystem

In farm HC, *V. nigripulchritudo* was neither detected in the water column nor in the renewal water. It was detected once in the sediment pore water, at low concentrations (<20 $CFU.mL^{-1}$) and late in the growout period (day 73). This strain was isolated, confirmed to be *V. nigripulchritudo* but tested non-virulent when injected to healthy *L. stylirostris* (table 1). In contrast, in farm DF, *V. nigripulchritudo* was detected in the sediment pore water also at low concentrations (ca. 33 $CFU.mL^{-1}$), but from day 11 on and until the onset of the mortality outbreak. Its concentration in the pore water increased during the disease outbreak (figure 2). It was also found in the water column on day 44, after its first detection in shrimp but short before the first observation of the mortality outbreak. It was then detected more frequently and at higher concentrations during the outbreak. It was detected only once in the renewal water, as a single colony on day 54, after the onset of the mortality. Thus in farm DF, both virulent and non-virulent strains were demonstrated in all compartments of the pond ecosystem (table 1). Regarding DF farm's surrounding environment, *V. nigripulchritudo* was only detected once in the renewal water, at a very low concentration and only after the onset of the mortality. This isolate was pathogenic, but former *V. nigripulchritudo* isolation from the pumping water only led to the isolation of three non-pathogenic strains (Goarant *et al.*, 2004). This detection, though rare and late, indicates that the pathogen could enter the system *via* the renewal water, but only at low concentrations. However, it was found earlier (from day 11 on), at higher concentrations and very frequently in the sediment pore water. The detection sequence in the affected farm (sediment at day 11, shrimp at day 40, pond water at day 44, renewal water at day 54) probably reflects the differences in abundance between the compartments and suggests that low concentrations of *V. nigripulchritudo* could survive in wet zones of the pond sediment during the dry-out period, then re-colonize larger zones of the sediment when the pond is filled up. Actually, there always exist wet zones of sediment in a 3-ha pond, even after long dry-out times, in which *Vibrio* strains would be able to persist. The overwintering at low levels in sediments was also described in *Vibrio parahaemolyticus* (Colwell, 1983). Shrimp ponds sediments are known as a rich medium prone to favour the growth of the *Vibrio* flora (Burford *et al.*, 1998; Kautsky *et al.*, 2000; La Rosa *et al.*, 2001). Actually, it seems that *V. nigripulchritudo* strains can easily find a place in the ecology of farm DF, whereas it remains at a very low level in farm HC. This suggests that some factors present in farm DF's ecosystem, and especially sediments, may favour *V. nigripulchritudo* as compared to farm HC's ecosystem. If so, a complete disinfection procedure (Bell and Lightner, 1992) of farm DF could help prevent the re-occurrence of the Summer Syndrome. Work is currently in progress to investigate if some sediment parameters in farm DF may favour the growth of *V. nigripulchritudo*.

Figure 2: Water temperature and presumptive *Vibrio nigripulchritudo* counts in pond water and sediment pore water in farm DF. The grey bar on X-axis represents the mortality period.
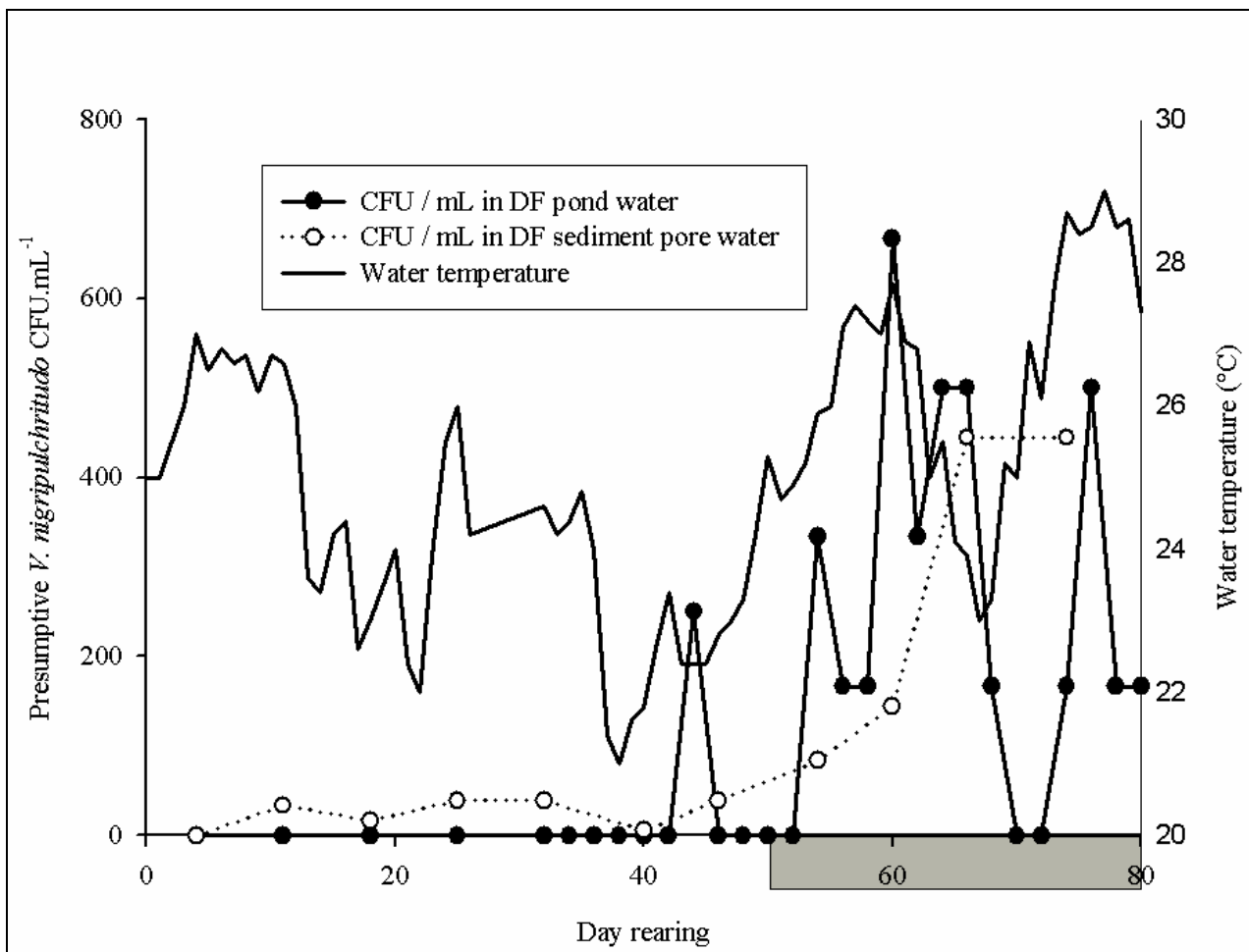
Lastly, the survey demonstrated a growth of planktonic crustaceans short before the onset of the epizootic in farm DF, evoking possible ecological relationships between *V. nigripulchritudo* and the zooplankton, as described for other *Vibrio* species, like *V. parahaemolyticus* (Kaneko and Colwell, 1975 ; Venkateswaran *et al.*, 1989) or *V. cholerae* (Huq *et al.*, 1983). Attempts to transform one pathogenic *V. nigripulchritudo* strain with a plasmid encoding the constitutive expression of Green Fluorescent Protein (GFP) are currently undertaken, that would permit the study of these relationships, as done with *V. cholerae* (Chiavelli *et al.*, 2001).

Comparison between farms

Very clear differences were observed between the two farms: In the healthy control farm (HC), *V. nigripulchritudo* was remaining at a low prevalence in shrimp until the end of the survey. Furthermore, all strains isolated before day 77 were non-pathogenic. It was detected only once and lately in the sediment. This infrequent detection means that *V. nigripulchritudo* was either absent or remained at low concentrations within HC farm's ecosystem during the survey. In contrast, in the affected farm (DF), both virulent and non-virulent *V. nigripulchritudo* were isolated from all compartments. From day 46 on, the rapid increase in prevalence suggests that either the phenomenon has turned to the epizootic mode, *i.e.* cross-contamination between infected (and/or moribund) and non-infected shrimp may occur or shrimp are exposed to high infective doses. This

ends up with the onset of the mortality outbreak, on day 50. Therefore, the Summer Syndrome can be considered as a disease that implicates environmental strains, as described in other shrimp vibriosis. An increase in population of the pathogenic *Vibrio* species was observed in our study, as also described with other studies on shrimp vibriosis (Lavilla-Pitogo *et al*., 1998; Sung *et al*., 1999). However, the contemporary detection of virulent and non-virulent strains in the ecosystem also suggests that a more complex "microbial ecology problem" could be one of the key factors in the Summer Syndrome.

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