Disease surveillance of cultured marine fish in the North of Bali, Indonesia

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Abstract. A regular surveillance of marine fish diseases was conducted from March to November 2019 in order to determine the occurrence time of the diseases within the mariculture centre of the North of Bali, Indonesia. The monthly surveillance was conducted by collecting 15 fish samples from each of the three hatcheries in Gerokgak and Penyabangan villages and of the two floating net cages in Pegametan Bay, Sumberkima village. Bacterial concentrations were grouped into 4 categories including low, moderate, high and very high. Surveillance data were analyzed using bivariate descriptive statistical methods. The results showed some important findings. First, the results of the study showed that NNV infection was found during the transitional seasons in March to June and September to November. Parasite infection were more frequent observed in fish with high and very high bacterial population. Second, high concentration of total bacteria in fish-feces occurred throughout the year. The prevalence of NNV infection and bacterial populations at the high to very high concentration were mostly occurred in the cultured fish in hatcheries at the size of 1-10 g, while in cultured fish at the net cages were mostly occurred at the size of $\geq 50$ g.

1. Introduction
Indonesia is one of the largest exporters of fishery products with a production target equivalent to an overall production increase of 353\% between 2009 and 2014 \cite{1}. One of the centers for grouper and sea bass hatchery and grow out in Indonesia is Gerokgak District, Buleleng Regency, Bali. In general, the potential land area for marine aquaculture in Buleleng was estimated at $\pm 1,050$ ha, and the area utilized for marine aquaculture was $227.3$ ha or 30.3\%, consisting of grouper floating net cages covering 30.5 ha, Milkfish 1.9 ha, sea bass 18.0 ha, seaweed 66.5 ha, and pearl oysters 110.4 ha. The potential for brackish aquaculture or ponds was 1,000 ha and has only been used for an area of 215.5 ha or 21.5\%, while freshwater aquaculture has potential for an area of 2,543 ha and has only been utilized for an area of 54.2 ha or 2.1\%. Grouper cultured commodities with an area of 50.0 Ha (8,600 units) has produced 378.6 tons \cite{2}.

Grouper culture business in Gerokgak District has been growing for 20 years. The hatchery business was carried out along the coastal waters of Gerokgak Village, Sanggalangit Village, Penyabangan Village and Musi Village, while the grouper grow-out business was carried out in Pegametan Bay, Penerusan Bay and Kaping Bay in Sumberkima Village. Marine fish farming in Gerokgak District continues to develop because of several economic advantages. This effort was
developing because the production process uses more existing coastal and marine resources, and uses a large local component, while the product has the potential to be exported [3].

The development of mariculture activities can also have a negative impact on the environment [4]. The use of artificial feed and fertilizers on plankton, as well as residual organic matter from rearing tanks or fish larvae are the main sources of pollution to the coastal waters [5]. In addition, water pollution can come from domestic waste, industrial waste treatment, household waste, livestock waste, and inland fisheries [6]. The presence of several types of bacteria in aquaculture waters is often used as an indicator of environmental pollution [7]. Total bacteria and Vibrio spp. in the coastal waters of Gerokgak District are more influenced by the season and aquaculture activities [8]. The high population of Vibrio spp. in coastal waters can affect the health of cultured fish [9], [8]. Fish can acquire pathogenic bacteria from contaminated aquaculture water or microbes that produce toxins in fish and can lead to unsafe fish consumption [10].

The incidence of parasitic, bacterial, and nervous necrosis virus (NNV) infections in Gerokgak District was reported throughout the year [11, 12]. Furthermore, it was reported that the prevalence of NNV virus infection was more common from March to July, and increased in November. The dominant type of parasite that infects marine fish in hatcheries is Trichodina spp., while in floating net cages is gill fluke (gill worms) [12], [13], [14]. Fish infected with the parasite have been reported to be infected with NNV at the same time. The prevalence of NNV virus infection tends to be followed by a pattern of increasing the total bacteria and Vibrio spp. [12]. Monitoring the incidence of disease in cultured fish at the marine fish farming center in Gerokgak District is carried out until 2019. The data obtained from these observations can be used as a basis for making decisions and actions needed in the future. The purpose of this study was to determine the occurrence time of the diseases within the mariculture centre of the North of Bali, Indonesia.

2. Methodology

2.1. Sampling location
Fish samples were taken from hatcheries and floating net cages of grouper (Epinephelus spp.) and sea bass (Lates calcarifer) in Gerokgak District, Buleleng Regency, Bali Province, Indonesia. The three locations selected were Gerokgak Village, Penyabangan Village, and Teluk Pegametan, Sumberkima Village. Fish sampling was carried out periodically every month from March to November 2019. Fish samples were taken from three hatcheries in Gerokgak Village and three hatcheries in Penyabangan Village, as well as two floating net cages in Pegametan Bay, Sumberkima Village. A total of 15 groupers or sea bass with a total length of 2.73-12.54 cm (average of 6.86±2.23 cm) were collected from each hatchery.

The fish sampled in the floating net cages were grouper and sea bass from the IMRAFE hatchery. The fish were stocked in floating net cages and kept for 3 months to monitor the health condition of the fish. Fish stocking was done in February, March and August. Each of 50 hybrid groupers and sea bass were cultured in two floating net cages (FNC) belonging to the Institute for Mariculture Research and Fisheries Extension (IMRAFE) and FNC belonging to local farmers. The fish were brought from the IMRAFE hatchery which had been treated with 100 ppm formalin for 1 hour to remove the parasites, and confirmed negative for NNV and iridovirus through PCR analysis. Each type of fish was cultured in one net (1x1x1 m²) and fed commercial pellets. Fresh water treatment and net replacement are carried out once a month in IMRAFE’s floating net cages and every 2 weeks in floating net cages owned by local farmers. Each of 10 groupers and 10 sea bass were sampled every month in both floating net cages (total length 10.78-20.03 cm with an average of 15.93 ±2.05 cm).

2.2. Performance of fish samples
Observations of fish performance were carried out on fish movement, skin color, and condition of the body surface, head, fins and tail. All fish samples were measured for length and body weight. Furthermore, fish samples were dissected to determine the condition of their internal organs.
2.3. Parasite observation
Observation of parasites by scraping the skin of fish with a cover glass, while observing the parasite on the gills by cutting the right and left gill lamellae. Both organs were placed on an object glass and observed under a microscope with a magnification of 40-400x.

2.4. Bacteria isolation
Total bacteria, Vibrio spp. and rod-shaped gram-negative bacteria isolated from fish feces by dissecting fish intestines. Each 3 fish in each hatchery and floating net cages were analyzed for bacteria. Bacteria were cultured in triptic soy agar (TSA), thiosulfate citrate bile salts-sucrose agar (TCBSA) and MacConkey agar, and incubated at 30°C for 24 hours. The number of bacterial colonies that grew was divided into 4 concentration groups. Total bacteria were divided into groups of low (< $10^5$ cfu/g), moderate ($10^5 - 10^6$ cfu/g), high ($10^7 - 10^8$ cfu/g), and very high ($10^9 - 10^{11}$ cfu/g) concentrations. While the number of Vibrio spp. and rod-shaped gram-negative bacteria were divided into four concentration groups including low (< $10^2$ cfu/g), moderate ($10^2 - 10^3$ cfu/g), high ($10^4 - 10^6$ cfu/g), and very high ($10^7 - 10^8$ cfu/g) concentrations. The calculation of the population group refers to the standards of the Pathology Laboratory at IMRAFE which has been accredited (ISO/IEC 17025:2017) and our previous report [12].

2.5. Virus detection with conventional PCR
Target organs such as eyes, brain or head were used for the detection of NNV, while the spleen and kidneys were used for the detection of iridovirus. Each target organ of five grouper or sea bass from each hatchery and floating net cage was collected for virus detection. The target organs of five fish samples were pooled into one microtube for analysis of NNV and iridovirus. Briefly, the RNA genome of the NNV virus was extracted using trizol according to the procedure [15]. The RNA genome was synthesized into cDNA with a reverse transcription system (Promega) kit. Samples were incubated at 70°C for five minutes, followed by 42°C for 60 minutes. The reaction was stopped by heating for five minutes at 95°C. The cDNA samples were stored at -20°C until use. NNV virus amplification was carried out following the amplification conditions previously reported by [16] using a pair of primers F2 and R3 [17].

Extraction of genomic DNA and DNA amplification conditions for iridovirus followed the procedure previously reported by [11], [12]. Genomic DNA amplification using GoTaq PCR Core System kit (Promega) with primers 1F (5'- CTCAAACACTCTGGCTCATC -3') and 1R (5'- GCACCAAACATCTC TATC -3'). Electrophoretic PCR results were read in gel agarose with 1xTAE buffer, observed with a UV transilluminator and documented with a gel camera.

2.6. Data analysis
Data from disease monitoring results were analyzed by bivariable descriptive statistics using online-based software “statulatorbeta” (http://www.statulator.com). The results of the analysis were presented in percentage form using the formula:

$$\% = \left( \frac{\text{Number of target fish}}{\text{Total number of fish}} \right) \times 100$$

3. Results and discussion
3.1. Disease surveillance in hatcheries
Fish samples obtained in hatcheries from March to November 2019 showed an average total length of 6.86±2.23 cm with an average weight of 6.05±4.83 cm (Figure 1). The highest fish length and weight were obtained in August (total length: 12.52±0.87 cm and weight: 24.23±6.83 g in Gerokgak H-1, and total length: 12.54±3.17 cm with body weight: 21.65±3.46 g in Pengabangan H-2). Nursery of fish in hatcheries to above the average size for sale or the size of fish being raised in
floating net cages (≥10 cm) may be due to not having a market/buyer or the fish are sick/deformity or something else.

![Graph of the average length (A) and weight of fish (B) from 6 hatcheries in North Bali coastal water.](image)

**Figure 1.** Graph of the average length (A) and weight of fish (B) from 6 hatcheries in North Bali coastal water.

Most of the fish observed in hatcheries were in healthy condition (Table 1). Sick fish were found almost every month with the highest percentage in August and November reaching 5.36%. The percentage of total sick fish was smaller (21.43%) compared to the percentage of healthy fish (78.57%). Sick fish showed symptoms of dark body color, skinny body, fin and tail drizzle, reddish wounds and ulcers in the lower abdominal area near the fins or anus. Incision of sick fish showed empty stomach and intestines with slightly yellowish fluid, slightly pale or reddish liver and slightly swollen spleen. Some fish samples also showed an open gill operculum, bent mouth, and bent body.

Parasite infestations in grouper and sea bass were found every month in the range of 1.79-10.71% (total 46.43%). The highest parasite infestation occurred in March and November up to 7.14-10.71%. NNV infections occurred in March to June and September to November with a range of 1.79-5.36% (total 19.64%). The highest NNV infection occurred in May and November (5.36%). Meanwhile, iridovirus infection was observed in March and April (1.79-5.36%) (Table 1).

Table 1 also showed that the high concentration of bacteria in fish feces (10^7 - 10^8 cfu/g) occurred from March to May, August. September and November with a range of 1.79-10.71% (total 37.50%). The population of bacteria with very high concentrations (10^9 - 10^11 cfu/g) occurred in April to July and October to November with a range of 1.79-10.71% (total 41.07%). Total Vibrio spp. with high concentrations (10^4 - 10^6 cfu/g) occurred in March to June and August to October with a range of 1.79-10.71% (total 44.64%). While the total Vibrio spp. with very high concentrations (10^7 - 10^9 cfu/g) occurred in June and July in the range of 8.93-10.71% (total 19.64%). Populations of rod-shaped gram-negative bacteria with high concentrations occurred in July and August with a range of 7.14-10.71% (total 17.86%).

The type of fish cultured in the hatchery in Gerokgak Village was the hybrid grouper (*Epinephelus fuscoguttatus* x *E. lanceolatus*), while the type of fish cultured in the Penyabangan Village is mostly hybrid grouper, and two other commodities such as sea bass (*Lates calcarifer*) and coral trout grouper (*Plectropomus leopardus*) (Table 2). Fish samples from three hatcheries in Gerokgak Village showed that the condition of sick fish (16.07%) was higher than that of sick fish (5.36%) in three hatcheries in Penyabangan Village. Similarly, fish infested with parasites in three hatcheries in Gerokgak Village was higher (30.36%) compared to fish infested with parasites (16.07%) in three hatcheries in Penyabangan Village. However, NNV and iridovirus infections in hatcheries in Penyabangan Village were higher (10.71% and 5.36%) compared to hatcheries in Gerokgak Village (8.93% and 1.79%). The total population of bacteria, *Vibrio* spp. and rod-shaped gram-negative bacteria in fish faeces varied and were similar in all hatcheries in both of Gerokgak and Penyabangan Villages. The high
percentage of parasitic infections in the hatchery in Gerokgak Village may be due to land conversion, waste from the hatchery itself and poor management of several hatcheries, causing an increase in organic and inorganic matter contamination in the coastal waters [12].

Table 1. Percentages of fish health condition and fish population infected by parasites, viruses, and bacteria every month in hatcheries.

| Date of sampling | Fish condition (%) | Population samples were positive infected by (%) | Total bacteria populations (%) |
|------------------|--------------------|-------------------------------------------------|--------------------------------|
|                  | Healthy | Sick | Parasite | NNV | Iridovirus | Low | Moderate | High | Very High |
| March            | 7.14    | 3.57 | 7.14     | 1.79 | 5.36       | 0   | 0        | 10.71| 0         |
| April            | 10.71   | 0    | 5.36     | 1.79 | 5.36       | 0   | 0        | 5.36 | 5.36      |
| May              | 10.71   | 0    | 3.57     | 5.36 | 0          | 1.79| 5.36     | 1.79 | 1.79      |
| June             | 8.93    | 1.79 | 5.36     | 0    | 0          | 0   | 3.57     | 0    | 7.14      |
| July             | 8.93    | 1.79 | 3.57     | 0    | 0          | 0   | 0        | 0    | 10.71     |
| August           | 7.14    | 5.36 | 1.79     | 0    | 0          | 0   | 3.57     | 8.93 | 0         |
| Sept.            | 8.93    | 3.57 | 5.36     | 1.79 | 0          | 0   | 7.14     | 5.36 | 0         |
| Oct.             | 10.71   | 0    | 3.57     | 1.79 | 0          | 0   | 0        | 0    | 10.71     |
| Nov.             | 5.36    | 5.36 | 10.71    | 5.36 | 0          | 0   | 0        | 5.36 | 5.36      |
| Total            | 78.57   | 21.43| 46.43    | 19.64| 7.14       | 1.79| 19.64    | 37.50| 41.07     |

| Date of sampling | Total Vibrio spp. (%) | Rod-shaped gram-negative bacteria (%) |
|------------------|------------------------|---------------------------------------|
|                  | Low | Moderate | High | Very High | Low | Moderate | High |
| March            | 0   | 3.57     | 7.14 | 0         | 7.14| 3.57     | 0    |
| April            | 5.36| 3.57     | 1.79 | 0         | 8.93| 1.79     | 0    |
| May              | 0   | 8.93     | 1.79 | 0         | 10.71| 0        | 0    |
| June             | 0   | 0        | 1.79 | 8.93      | 8.93| 1.79     | 0    |
| July             | 0   | 0        | 0    | 10.71     | 0   | 0        | 10.71|
| August           | 3.57| 3.57     | 5.36 | 0         | 1.79| 3.57     | 7.14 |
| Sept.            | 0   | 1.79     | 10.71| 0         | 10.71| 1.79     | 0    |
| Oct.             | 0   | 0        | 10.71| 0         | 10.71| 0        | 0    |
| Nov.             | 0   | 5.36     | 5.36 | 0         | 5.36| 5.36     | 0    |
| Total            | 8.93| 26.79    | 44.64| 19.64     | 64.29| 17.86    | 17.86|

Note:
Bacteria populations
- $<10^2$ cfu/g : Low
- $10^2$-$10^6$ cfu/g : Moderate
- $10^6$-$10^{10}$ cfu/g : High
- $10^{10}$-$10^{11}$ cfu/g : Very High
Total Vibrio spp. and rod-shaped gram-negative bacteria
- $<10^2$ cfu/g : Low
- $10^2$-$10^6$ cfu/g : Moderate
- $10^6$-$10^{10}$ cfu/g : High
- $10^{10}$-$10^{11}$ cfu/g : Very High
Table 2. Percentages of fish condition associated with parasitic infestation, viral infections and bacterial concentrations based on sampling location

| Sampling location       | Fish Species                  | Fish condition (%) | Population samples were positive infected by (%) |
|-------------------------|-------------------------------|-------------------|-----------------------------------------------|
|                         | Sea bass (Lates Calcarifer)   | Healthy           | Parasite                                       |
|                         | Hybrid grouper (Epinephelus spp.) | Sick             | NNV                                           |
|                         | Coral Trout grouper (Plectropomus leopardus) | Parrotfish        | Iridovirus                                    |
| Gerokgak Village        | 0                             | 32.14             | 30.36                                         |
| Penyabangan Village     | 1.79                          | 16.07             | 8.93                                          |
| Total                   | 1.79                          | 46.43             | 19.64                                         |

| Sampling location       | Total bacteria populations (%) | Total Vibrio spp. (%) | Rod-shaped gram-negative bacteria (%) |
|-------------------------|--------------------------------|-----------------------|--------------------------------------|
|                         | Low  | Mode-rate | High  | Very High | Low  | Mode-rate | High  | Very High | Low  | Mode-rate | High  |
| Gerokgak Village        | 1.79 | 10.71     | 12.50 | 23.21     | 0    | 12.50      | 26.79 | 8.93      | 26.79| 12.50      | 8.93  |
| Penyabangan Village     | 0    | 8.93      | 25.00 | 17.86     | 8.93 | 14.29      | 17.86 | 10.71     | 37.50| 5.36       | 5.36  |
| Total                   | 1.79 | 19.64     | 37.50 | 41.07     | 8.93 | 26.79      | 44.64 | 19.64     | 64.29| 17.86      | 17.86 |

Fish samples with a size of 1-5 grams had a higher susceptibility to NNV and iridovirus infections (12.5% and 5.36%) compared to fish with a size of 6-10 grams (1.79%) and sizes 11-31 grams (1.79% and 0) (Table 3). Similarly, the total population of bacteria, Vibrio spp. and rod-shaped gram-negative bacteria at high and very high concentrations were found with higher percentages in the fish population with a size of 1-5 grams, followed by fish with a size of 6-10 grams and 11-31 grams. Parasite infestation in fish size between 1-5 grams and 6-10 grams had a higher percentage (16.07% and 17.86%) compared to fish with size of 11-31 grams (12.50%).

Table 3. Percentages of fish-size (gram) associated with parasitic infestation, viral infections and bacterial concentrations

| Fish size (gram) | Population samples were positive infected by (%) | Total bacteria populations (%) | Total Vibrio spp. (%) | Rod-shaped gram-negative bacteria (%) |
|------------------|-----------------------------------------------|--------------------------------|-----------------------|--------------------------------------|
|                  | Parasite | NNV | Iridovirus | High | Very High | High | Very High | High | High |
| 1-5              | 16.07     | 12.50 | 5.36        | 14.29 | 21.43     | 19.64 | 12.50      | 12.50 |
| 6-10             | 17.86     | 1.79  | 1.79        | 17.86 | 10.71     | 16.07 | 3.57       | 3.57  |
| 11-31            | 12.50     | 5.36  | 0           | 5.36  | 8.93      | 8.93  | 3.57       | 1.79  |
| Total            | 46.43     | 19.64 | 7.14        | 37.50 | 41.07     | 44.64 | 19.64      | 17.86 |

Fish in hatcheries that were infested with parasites (46.43%) or not infected with parasites (53.57%) were seen to contain NNV virus in their bodies with low percentages (5.36% and 14.29%), as well as to iridovirus infection (3.57%) (Figure 2A). The parasite-infested fish showed a slightly higher population of bacteria with high (17.86%) and very high (23.21%) concentrations compared to fish that were not infested with parasites (19.64% and 17.86%). Percentage of Vibrio spp. in fish feces was seen higher at high concentrations in fish infested with parasites (23.22%) and fish not infested with
parasites (21.43%). Parasite infestations were found in low intensity (<10 parasites/fish) in healthy fish because the farmers had treated them with chemicals or fresh water if the fish had decreased appetite and slowed movement.

The total sample of fish (19.64%) that were positive for NNV infection showed a smaller number of parasites (5.36%) than the fish that were not infected with the parasite (14.28%) (Figure 2B). A total of 1.79% of fish that were positively infected with NNV were also found to be infected with iridovirus. This case shows that fish cultured in hatcheries can be infected with two viruses at once. Most of the fish infected with NNV contained a total population of bacteria with a high concentration of 7.14% and a very high concentration of 8.93%. The NNV-infected fish also had a total of Vibrio spp. with a low concentration of 3.57%, and the number increased at moderate (7.14%) and high (8.93%) concentrations. However, fish infected with NNV were only found to have rod-shaped gram-negative bacteria with low (14.29%) and moderate (5.36%) concentrations. An increase in the number of bacteria and Vibrio spp. were also shown by fish that were not infected with NNV. This indicates that NNV infection in cultured fish in hatcheries tends to be unrelated to the concentration of the bacterial population in the fish gut as well as parasitic infestation.

![Figure 2](image)

**Figure 2.** Percentages of fish infested with parasite (A), and fish infected with NNV associated with other infections.

### 3.2. Disease surveillance in floating net cages

Hybrid grouper cultured in two floating net cages in Pegametan Bay, Sumberkima Village has an initial total length range of 12.27-14.77 cm (an average of 13.76±1.03) with a body weight of 26.95-63.85 g (an average of 44.95±14.41 g). While the sea bass cultured in two floating net cages had an initial total length of 10.78-16.70 g (an average of 14.27±2.10 g) with a body weight of 14.41-70.14 g (an average of 40.34±20.10 g). The hybrid grouper and sea bass cultured in the two floating net cages grew for 3 months (Figure 3). The hybrid grouper showed increase in total length of 2.19±0.88 – 5.60±0.58 cm with a body weight of 31.52±2.95 – 59.08±9.34 g, while the sea bass showed increase in total length ranging from 2.80±1.45 – 3.62±0.18 cm with an average body weight ranging from...
31.16±6.75 – 38.7±18.78 g. The highest fish growth occurred in cultured from September to November.

Figure 3. The average length (A) and body weight (B) of hybrid grouper and sea bass cultured in floating net cages (FNCs) at Pegametan bay

Hybrid grouper and sea bass cultured in floating net cages showed a healthy condition (100%) for 9 months of observation. However, both types of fish showed parasite infestations and detected the presence of NNV and iridovirus in the eyes, brain and spleen (Table 4). The fish samples were 100% (11.11%/month) infested with parasites either on the body surface or in the gill lamellae. NNV infections were detected in March to June and September with a percentage of 2.78-8.33%, while iridovirus infections were detected in June (5.56%) and August (2.78%). Fish samples also contained high and/or very high concentrations of bacteria in their intestines every month for 9 months (5.56-11.11%/month). Total percentage of Vibrio spp. with high concentrations observed in March, April, July, August and October with a percentage of 2.78-5.56% (25-50% of the sample observed per month). The highest concentration of rod-shaped gram-negative bacteria was found in May at 2.78%.

Table 4. Percentages of fish condition and fish population infected by parasites, viruses, and bacteria every month in floating net cages

| Date of sampling | Healthy fish (%) | Population samples were positive infected by (%) | Total bacteria populations (%) | Total Vibrio spp. (%) | Rod-shaped gram-negative bacteria (%) |
|------------------|-----------------|-----------------------------------------------|-------------------------------|----------------------|-------------------------------------|
|                  | Healthy fish (%) | NNV Low | NNV High | Iridovirus Low | Iridovirus High | Very High | Low | Moderate | High | Low | Moderate | High |
| March           | 11.11           | 11.11   | 2.78     | 0              | 0                  | 11.11     | 0   | 5.56     | 5.56 | 0   | 11.11     | 0    |
| April           | 11.11           | 11.11   | 5.56     | 0              | 0                  | 11.11     | 2.78| 5.56     | 2.78 | 11.11| 0          | 0    |
| May             | 11.11           | 11.11   | 8.33     | 0              | 0                  | 5.56      | 5.56| 5.56     | 5.56 | 0   | 5.56      | 2.78 |
| June            | 11.11           | 11.11   | 2.78     | 5.56           | 0                  | 11.11     | 0   | 0        | 11.11| 0   | 0          | 0    |
| July            | 11.11           | 11.11   | 0        | 0              | 0                  | 11.11     | 2.78| 2.78     | 5.56 | 0   | 11.11     | 0    |
| August          | 11.11           | 11.11   | 0        | 2.78           | 0                  | 11.11     | 0   | 5.56     | 2.78 | 2.78| 11.11     | 0    |
| Sept.           | 11.11           | 11.11   | 5.56     | 0              | 2.78               | 8.33      | 3.33| 2.78     | 0    | 11.11| 0          | 0    |
| Oct.            | 11.11           | 11.11   | 0        | 0              | 0                  | 11.11     | 0   | 8.33     | 2.78 | 2.78| 8.33      | 2.78 |
| Nov.            | 11.11           | 11.11   | 0        | 0              | 0                  | 11.11     | 0   | 11.11    | 0    | 11.11| 0          | 0    |
| Total           | 100             | 100     | 25       | 8.33           | 5.56               | 47.22     | 47.22| 36.11    | 44.44| 19.44| 69.44     | 27.78|
Hybrid grouper and sea bass cultured in two floating net cages had the same susceptibility to parasite infestation. This has been shown by the total parasite infestation of up to 100% for 9 months of observation. NNV infections were more frequent in cultured fish at floating net cages-IMRAFE (13.89%) compared to cultured fish at floating net cages-local farmer (11.11%). On the other hand, iridovirus-infected fish were more frequent in cultured fish at floating net cages-local farmer (5.56%) compared to cultured fish in floating net cages-IMRAFE (2.78%) (Table 5). Hybrid grouper and sea bass cultured in both floating net cages also showed a higher total bacterial population at high and very high concentrations (47.22%). However, the population of Vibrio spp. and rod-shaped gram-negative bacteria showed higher numbers at low and moderate concentrations (36.11-44.44% and 69.44-27.78%). These results indicate that the bacterial population found in the digestive tract (intestine) of grouper and sea bass cultured in floating net cages were not entirely Vibrio spp. and rod-shaped gram-negative bacteria.

### Table 5. Percentages of fish cultured in floating net cages (FNC) associated with parasitic infestation, viral infections and bacterial concentrations in fish-intestine.

| Sampling location       | Population samples were positive infected by (%) | Total bacteria populations (%) | Total Vibrio spp. (%) | Rod-shaped gram-negative bacteria (%) |
|-------------------------|-------------------------------------------------|--------------------------------|-----------------------|---------------------------------------|
|                         | Parasite NNV Irido-virus Low High Very High      | Low Moderate High             | Low Moderate High     | Low Moderate High                      |
| FNC-IMRAFE              | 50 13.89 2.78 5.56 19.44 25                    | 16.67 19.44 13.89 36.11 13.89 0 |
| FNC-local farmer        | 50 11.11 5.56 0 27.78 22.22 19.44 25 5.56 33.33 13.89 2.78 |
| Total                   | 100 25 8.33 5.56 47.22 47.22 36.11 44.44 19.44 69.44 27.78 2.78 |

Hybrid grouper and sea bass had the same prevalence of parasite infestation (Figure 4A). Based on the total number of fish infected with NNV (25%), white snapper showed a higher percentage of NNV infection (13.69%) than the hybrid grouper. On the other hand, from a total of 8.33% of fish infected with iridovirus, hybrid grouper had a higher probability of being infected with iridovirus (5.56%) compared to sea bass (2.78%). Percentage of total bacteria, Vibrio spp. and rod-shaped gram-negative bacteria from both types of fish showed almost the same number at all concentration levels.
Fish samples with a size of >50 grams showed a higher probability of being infected with parasites and NNV (33.33-55.56% and 5.56-19.44%) compared to smaller fish (11-20 g: 11.11% and 0%) (Figure 4B). While the percentage of fish infected with iridovirus was the same (2.78%) in all sizes of fish. Total bacteria at high and very high concentrations were seen with higher percentages in fish with a size of 21-50 g (11.11% and 19.44%) and fish size >50 g (25% and 27.78%). Total Vibrio spp. seen with a higher percentage at low and moderate concentrations in all sizes of fish with a total of 36.11% and 44.44%, respectively. Likewise, rod-shaped gram-negative bacteria showed large numbers at low concentrations with a total of 69.44% and moderate concentrations with a total of 27.78%. These results indicate that the bacterial population increases with the size of the fish.

Parasitic-infested fish (100%; Figure 5A) showed a lower percentage of NNV and iridovirus infection (25% and 8.33%) compared to those that did not contain NNV virus (75%) and iridovirus (91.67%) in their bodies. The fish were seen to have a high percentage of bacterial population in the high and very high concentration groups (47.22%). however, the fish had a percentage of Vibrio spp. and rod-shaped gram-negative bacteria in the low and moderate concentrations were higher than in the high concentration group.

The results of the descriptive analysis of NNV-infected fish against parasite infestations showed that 75% of the parasite-infected fish were not infected with NNV, while the other 25% were infected by NNV. However, there was no indication of fish samples being infected with NNV virus and iridovirus at the same time (Figure 5B). From 25% of fish infected with NNV showed an increase in the total concentration of bacteria from low (5.56%), high (36.11%) and very high (58.33%). An increase in the number of bacteria was also shown by fish that were not infected with NNV. However, the increase in the total percentage of bacteria was not followed by an increase in the concentration of Vibrio spp. and rod-shaped gram-negative bacteria. This indicates that NNV infection in fish cultured in floating net cages may not be associated with increased concentrations of total bacteria or Vibrio spp. and rod-shaped gram-negative bacteria.

**Figure 5.** Percentages of fish infested with parasite (A) and fish infected with NNV (B) associated with others infections

Fish health surveillance was an activity used to identify a disease incidence including distribution and prevalence of disease. Surveillance was used to provide information on disease incidence in an area for control programs to reduce the risk of spreading aquatic animal diseases through national,
Several infectious diseases have been reported to cause mass mortality in grouper and sea bass cultured in Indonesia, such as NNV [20], an iridovirus known as grouper sleepy iridovirus (GSDIIV) [21], Vibrio spp. [22] and ectoparasites [12], [13], [14]. Monitoring the prevalence of viral infections in North Bali in 2017 reported the incidence of NNV infection in marine fish cultured in hatcheries almost every month with the highest prevalence rate in September, and the highest iridovirus infection occurred in August [11]. Furthermore, it was reported that the highest prevalence of NNV in marine fish cultured in floating net cages occurred in October, and the highest iridovirus infection occurred in February. In monitoring the incidence of viral infections in 2018, the prevalence of NNV infections was reported to be more frequent from March to July, and increased in November. Meanwhile, iridovirus infection was only found in October [12]. The results of surveillance on the incidence of viral diseases in 2019 had almost the same pattern of occurrence as in the previous year, such as NNV infections in fish cultured in hatcheries occurred in March to June and September to November, while iridovirus infection was observed in March and April. NNV infections in fish cultured in floating net cages were detected in March to June and September, while iridovirus infections were detected in June and August. The results of surveillance on the incidence of viral diseases for 3 years indicate that NNV infections occur almost throughout the year and the possibility of the virus is always present in the waters and cultured marine fish in North Bali. Surveillance results also show that iridovirus infection occurs at certain times, but the iridovirus infection is also an obstacle in marine fish farming in North Bali because it can cause mass mortality.

The presence of NNV strains in regional Asia may indicate that hatcheries were at risk from NNV not only from external fish fry but also from endemic reservoirs [23]. The use of fish eggs and fish seeds from various sources has the potential to increase the horizontal transmission of NNV virus in hatcheries [24]. Meanwhile, NNV infection in floating net cages that are directly related to the marine environment was influenced by several variables such as juvenile sources, weather changes, technician experience, DO water, and feed. Trash feed could be a source of transmission from VNN [25]. Water temperature also affects the incidence of NNV infection in the waters of North Bali. The decrease in water temperature in April-June and September-November affects the increase in disease incidence in cultured fish [11], [12].

Surveillance results showed that fish cultured in hatcheries and floating net cages were susceptible to parasites, of which 3.57-25% were also detected to contain NNV and iridovirus genes. Parasite-host interactions are influenced by environmental changes, especially habitat degradation by anthropogenic pollutants and climate-induced oceanographic alterations. The association depends on the susceptibility and resistance of the fish [26]. The fish stocking density was not the main factor in the process of spreading parasites in waters, but rather the interaction between the environment, fish and parasitic pathogens [27]. Joint infection of several pathogenic microorganisms was common in nature and often involves parasites [28]. It was further reported that parasites on the immune system caused immunodepression and cytokine effects. The interaction of two pathogens was synergistic or antagonistic and results in an increase or inhibition of one or both pathogens, increasing or decreasing the severity of the disease. These interactions have an important impact on disease progression and severity [29]. Parasitic infections with concurrent viral infections were also found in grouper and sea bass in marine fish farming centres in North Bali in the previous year [12]. Parasite infestation was suspected to affect fish health and can be a trigger for secondary infections such as viruses or bacteria.

In this study, parasite infestation was found more frequently observed in fish with high and very high bacterial population. The same case was also reported in grouper which was a moderate to strong
association between *Photobacterium damselae* and iridovirus with the *Vibrio vulnificus*, and between NNV and *V. alginolyticus* in sea bass [30].

Infection with two viruses at the same time was found in groupers in hatcheries and floating net cages. Fish that were detected by NNV and iridovirus viruses were still mostly healthy [12]. The condition of clean waters and adequate nutritional feed may cause these fish to be resistant to VNN and iridovirus infections. In addition, the treatment of several chemicals and antibiotics given by the farmers resulted in healthy fish even though they were positive for the virus. The fish may get sick when under stress. As reported in wild infected fish without clinical symptoms it can show pathological changes under stressful conditions. Fish under stress can play an important role in the multiplication and spread of the virus. Fish as infection reservoirs can spread pathogens as virus carriers [31].

Larval and juvenile stages of sensitive species to NNV infection [32]. These findings were similar to our findings in this study in which the prevalence of NNV infection and bacterial concentrations in fish size 1-5 g was higher compared to groups of fish size 5-10 g and 11-31 g. The high concentration of bacteria in small fish in hatcheries was due to the fact that there was less water change in the rearing tank for small fish than for larger fish. The high population of bacteria in the waters can affect the health of cultured fish [9]. Meanwhile, the prevalence of NNV infection and bacterial concentrations in floating net cages was found to be higher in fish >50 grams. The fish have been cultured in floating net cages for 2-3 months until they reach a size of >50 g where they have been exposed to a direct aquatic environment. Similar results were also reported in rainbow trout (*Oncorhynchus mykiss*) where cumulative mortality from IHNV (Hematopoietic Necrosis Virus) infection in 1 g fish was higher than the 8 g and 25 g fish groups [33]. However, changes in the susceptibility of fish of different sizes among the progenies. Small tilapia fish were also reported to be more susceptible to tilapia lake virus (TLV) infection. Although multiple factors such as environmental, farm management practices, strains of virus also contribute to the different susceptibility of fish to viral infection [34].

4. Conclusion
The results showed that NNV infection was found during the transitional seasons in March to June and September to November. Iridovirus infection was observed in March and April in hatcheries, and in June and August in fish cultured in floating net cages. Parasitic infestation was found throughout the year and more frequently observed in fish with high and very high bacterial population. The prevalence of NNV and bacterial populations at the high to very high concentration were mostly occurred in the cultured fish in hatcheries at the size of 1-10 g, while in cultured fish at the net cages were mostly occurred at the size of ≥ 50 g.

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