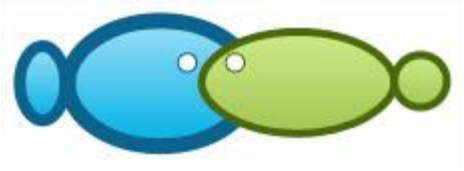

The mass death of Nile tilapia (*Oreochromis niloticus*) in Sorong District, West Papua, Indonesia

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Abstract. The mass death in tilapia fish farming, in Sorong District in 2018, caused considerable losses. This study aimed to identify the cause of mass mortality in tilapia fish farming in Sorong District. Fish samples were taken from 4 sub-districts with nine sampling locations in Sorong district, the samples were then isolated from bacteria. The obtained bacterial isolates were characterized and biochemically tested for bacteria. As a result, 20 pure bacterial isolates were obtained. The results of the characterization of bacterial isolates showed that all isolates were Gram-negative and oxidation-positive. 11 of them were catalase-positiveand 9 were catalase-negative. Indol test reaction showed all were positive, except the SMA isolates. Bacterial carbohydrate fermentation test results showed all isolates are positive, excepted glucose SMA isolates. 12 isolates are lactose-positive and 8 negative. Bacterial isolates were obtained at densities of 10-12 CFU mL^{-1}, in live fish samples taken from several ponds, there were several bruises and several fish in the pond died. One characteristic of bacterial infection is the presence of ulcers in fish. The results demonstrate that the fish mortality in Sorong District was caused by *Aeromonas hydrophila* infection.
Key Words: identification, disease, bacteria, characterization, *Aeromonas hydrophila*.

Introduction. The aquaculture sector has an important role for human food production, due to the high nutrient content of fish. Fish is a source of protein, which is essential for the human health (Baldissera et al 2018). Aquaculture growth worldwide is counter-balanced by infections and spread of disease. In Indonesia, the resulting losses amounted 3 million dollars per year (Kang et al 2013). The occurrence of mass deaths in Nile tilapia (*Oreochromis niloticus*) fish ponds in Malaus village, Malaus sub-district, Sorong District, in 2018, caused considerable losses. The clinical symptoms suggested that an infectious disease could be the cause. *O. niloticus* is one of the most cultured fish species in West Papua and the demand growths by 12.2% yearly (Fadl et al 2017).

The emergence of a disease in the cultivation unit can have various reasons, including environmental conditions and pathogenic bacteria. Elucidating the cause of fish death or health deterioration is crucial (Stride et al 2014). Among the most dangerous diseases are infections caused by bacteria, viruses, and parasites. *Aeromonas hydrophila* is a bacterium causing lethal Motile Aeromad Septicemia (MAS). The main symptoms are: tissue tear, bleeding, ulcers, swelling, and rupture of blood vessels (Ling et al 2016). In the early 2000s, there were reported and successfully isolated pathogenic bacteria attacking eel, namely *Vibrio vulnificus*, *Edwardsiella tarda*, and *Aeromonas* bacteria. Currently, a new disease emerged due to *Shewanella putrefaciens* and *Shewanella xiamenensis* (Esteve at al 2017). In salmon, *Aeromonas salmonicida* caused furunculosis (Menanteau-Ledouble & El-Matbouli 2016).

The current research was conducted to describe the causes of mass death of fish that occurred in Malaus Village, Sorong District. Identification was done by purifying bacterial isolates and biochemically tested.

Material and Method

Sampling location and methodology. According to Suman et al (2014), South Papua (Sorong District) is an area rich in demersal fish resources such as shrimp, red snapper, white snapper, bawal, stingray, milk shark and also small pelagic fish such as teri, tuna, bloated and other fish groups such as grouper, napoleon, lobster, and ornamental fish. In general, climate and weather conditions in Sorong district range between 20-38^{0}C, with a temperature change of 2^{0}C per year, wind speeds ranging from slow to moderate, between 8 and 15 m sec^{-1}. The study was conducted based on information from the District Fisheries Service of Sorong, where there was a mass death of *O. niloticus* in one of the ponds from Malaus Village, due to an unknown disease. The collected information allowed retracing the distribution of the fish farming units across the Sorong district. The research location can be seen in Figure 1.

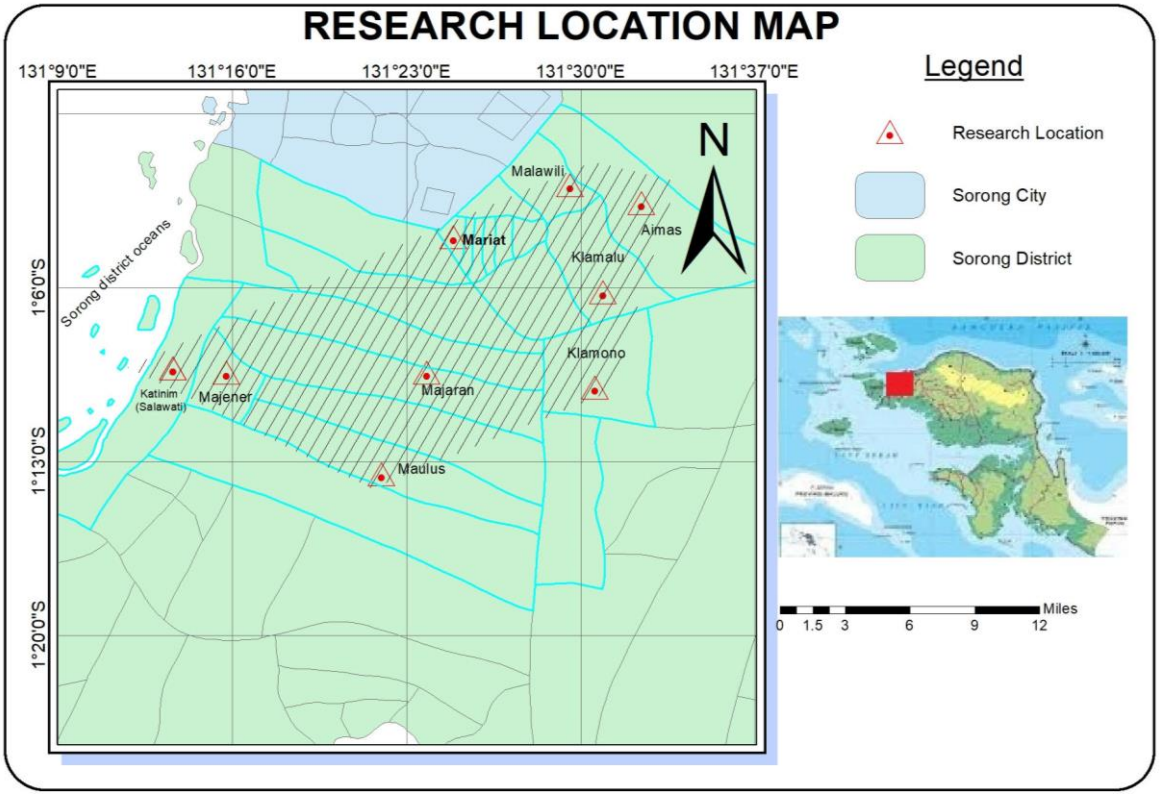

Figure 1. Sampling location.

Samples consisted in five *O. niloticus* specimens randomly chosen from the pond. Water quality data were collected from each location. Samples were put into a bag filled with water and labeled, at each location.

Sampling locations selection was based on interviews and discussions with the head of the Sorong District Maritime Affairs and Fisheries Office. They were located in 4 sub-districts, namely Salawati, Mariat, Aimas, and Klamono. These four sub-districts are producing aquaculture production in Sorong District. Twenty samples were collected from the above mentioned areas. Salawati is a sub-district that cultivates more fish compared to the other sub-districts in the district of Sorong, so that the majority of samples were taken in Salawati sub-district by five pieces. Water quality parameter testing was also carried out to support research data. Water quality parameters tested were brightness,

temperature using a water thermometer, pH using pH pen (CT-6023 Kedida, China), ammonia, nitrate, nitrite and DO using reagent kit (water test kit). Nitrite test was carried out by taking 5 mL of water sample and placing it into a reaction bottle. Afterwards, 5 drops of reagent 1 was added and shaked until homogeneous. 5 drops of reagent 2 were added and shaked until homogeneous. After 5 minutes the color was matched with the color indicator.

Bacterial isolation. Bacterial isolation was carried out at the Sorong Fish Quarantine and Quality Control Station. The bacteria were isolated from fish gills by aseptically opening the gill cover and then cutting fins with scissors and putting them in a sterile test tube. Homogenization was carried out by vortexing and was diluted 12 times, dilutions 10, 11, and 12 were planted in Petri dishes. Each Petri dish was labeled at the sampling location. Bacterial isolation was carried out by taking isolates with the highest value of CFU mL^{-1} for the bacterial colonies found in the last three dilutions. Then, the isolates were purified by re-striking the Petri dish, in order to obtain pure bacterial isolates ready to be planted on sloping agar (Tariq et al 2016).

Bacterial characterization. The bacteria planted on agar media (TSA) produced 30 bacterial isolates, which were then purified into 20 bacterial isolates, in several steps. Thirty bacterial isolates were characterized through the Gram staining test. A catalase test was carried out by dripping 3% hydrogen peroxide on the bacterial colony (Wu et al 2012). The tryptopan enzyme activity test was carried out by dripping the Kovacs reagent. Only isolates or colonies that had the same regular cell shape were continued to the next stage (Boulares et al 2012). From this operation 20 bacterial isolates resulted with separate cell forms.

Bacterial biochemistry test. The obtained pure bacterial isolates, once morphologically characterized, were then biochemically tested, as the initial stage of the bacterial identification process. Bacterial identification was carried out to ascertain the cause of death, by inclusuvely using the standard biochemical tests (Whitman et al 2012). Bacterial isolates were rejuvenated to obtain a better bacteria growth performance. The catalase test was carried out by taking a colony and dripping H_2O_2 (Boubekri & Ohta 1996). The characteristic reaction of the biochemical tests carried out is the carbohydrate fermentation, including glucose, lactose, arabinose, glucose, inositol, mannitol, maltose, trehalose, xylose, sorbitol, dulsitol, and sucrose (Boubekri & Ohta 1996). Chemical reaction tests were carried out to observe which enzymes were activated by bacteria which were indicated by changes in color. This was carried out with triple sugar iron (TSI) Agar, lysine iron agar (LIA), arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, Simmons citrate, H_2S, urease, indole, MR, VP, TCBS Mac Konkey, Muler Hinton broth, phenylalanin deaminase, aesculin, and gelatin (Sakai et al 1993; Khan & Ghosh 2012). The test was carried out by inserting and adding isolates into the test solution available in each test tube. Among the tests, there were chemical preparations, such as: indol, MR, VP, and ornithine (Pandolfi et al 2007).

Carbohydrate fermentation test. The ability of bacteria to hydrolyze carbohydrate members is an indicator of bacterial type, namely aerobic or anaerobic (Reddick 1975). Carbohydrate derivatives used to test are glucose, lactose, arabinose, inositol, mannitol, maltose, trehalose, xylosa, sorbitol, dulcitol, and sucrose.

Morphological character of bacteria. The results of dilutions 10, 11, and 12 in the bacterial isolation process resulted in 30 isolates, which were then purified into 20 isolates. The isolates were then subjected to morphological and gram tests (purple crystals and safranin), using a microscope (CX23 Olympus, Japan) (Claus 1992). The

parameters observed included the color, shape and edge of the colony, the elevation, the cell shape, color, and the Gram type (Brucker 1986). Also, gram and oxidative fermentative (O/F) agar tests were used to determine the β-hemolytic which showed the clear zone around the colony or lysis completely, that can be indicated from cytochrome oxidase, and catalase (Sakai et al 1993). Eye observations of bacterial colonies were performed for morphological and Gram tests. The tests results showed that all isolates had Gram (-) and all the colony types shared the following characteristics: color (beige), colony shape (rounded), edge colony (flat), elevation (convex), cell shape (stem), cell color (pink). However, SMA isolates had a white colony.

Results and Discussion. *O. niloticus* mortality in Sorong District is presumed to be caused by infectious diseases (pathogenic bacteria, viruses, fungi, protozoa, and parasites) and non-infectious diseases (i.e. unappropriate environmental conditions or water pollution). More than 30 pathogenic bacteria have the potential to cause diseases in aquatic biota (Carrias et al 2012). New sources of disease are due to climate change or limited water resources, requiring extra handling efforts to maintain fish health (Assefa & Abunna 2018). The vulnerability to the *O. niloticus* pathology is exacerbated by the environmental stress. The water quality parameters showed the following average values: pH of 7.025, temperature of ±30^{0}C, brightness of 26.6%, oxygen of 3.775 mg L^{-1}, nitrate of 6 mg L^{-1}, nitrite of 0.6 mg L^{-1} and ammonia of 0.925 mg L^{-1}. The water quality measurement results can be seen in Table 1. Aquaculture quality is strongly influenced by the location. In the laboratory, the optimal water quality for tilapia had the following parameter values: pH of 6.29, temperature of 26.53^{0}C, DO of 9.00 mg L^{-1}, brightness of 100%, ammonia of 0.08 mg L^{-1}, nitrite of 0.01 mg L^{-1} and nitrate of 0.46 mg L^{-1} (dos Santos et al 2019). The research results of Yilmaz (2019) revealed that the optimal quality of water in the tilapia culture in tubs has the following parameter values: temperature of 27^{0}C, pH of 7.32, DO of 7.5 mg L^{-1}, ammonia of 0.015 mg L^{-1}, nitrite of 0.022 mg L^{-1} and nitrate of 0.10 mg L^{-1}. Water quality for fish culture in ponds has the following parameter values: pH of 6.5-9, temperature of 25-39^{0}C, brightness of 30-40%, DO of 3-5 mg L^{-1}, nitrite of 0.5-2.5 mg L^{-1} and ammonia of 0.7-2.4 mg L^{-1} (Boyd 1990). Therefore, it can be concluded that the water quality at the study site was still in the normal ratio and was suitable for aquaculture production.

Table 1

Water quality parameter

Test parameter	*Sub-district*			
	Salawati	*Mariat*	*Aimas*	*Klamono*
pH	7.1±0.6	7.1±0.2	6.8±0.7	7.5±0.0
Temperature (ºC)	29.3±0.5	28.5±0.5	29.8±0.4	30.0±0.0
DO (mg L^{-1})	3.9±0.6	3.5±0.4	3.8±0.5	4.0±0.0
Brightness (cm)	25.0±5.8	27.8±7.5	28.2±2.4	30.0±0.0
Nitrate (mg L^{-1})	6.5±2.4	6.3±2.5	5.0±0.0	5.0±0.0
Nitrite (mg L^{-1})	0.7±0.2	0.5±0.0	0.6±0.2	0.5±0.0
Ammonia (mg L^{-1})	0.9±0.5	0.9±0.25	1.0±0.0	1.0±0.0

Biochemical test results showed that all isolates were Gram-negative, coccus-shaped. All the indole and MR tests were positive isolates. SMA isolates were VP-negative. Citrate test gave 12 positive isolates and 8 negative isolates. The carbohydrate fermentation test showed that negative SMA isolates did not react. The sucrose test showed 7 negative isolates and 13 positive. Other 12 isolates were positive and 8 were Dulcitol-negative. Lactose showed 8 negative isolates and 12 positive.

The results of bacterial biochemical tests are presented in Table 2 and 3 for the key parameters used to identify bacteria, according to Whitman et al (2012). The bacterial biochemical tests included catalase tests and oxidative/fermentative tests.

These tests are used to explain the ability of bacteria to metabolize carbohydrates by oxidative, fermentative or non-saccharolytic pathways, a signature of bacteria in the culture media (Public Health England 2019). Test results showed that all isolates were Gram negative, this was an indication of β-hemolytic, which can oxidize cytochrome (Sakai et al 1993). The methyl red (MR) and Voges-Proskauer (VP) tests were synchronized with IMVIC (indole, MR, VP, and citrate). MR test is an indicator of bacteria producing acid via the fermentative path. VP aims to detect bacterial production of acetoin (acetyl methyl carbinol or 3-hydroxybutanone) (Mcdevitt 2009). Only one isolate was indole-negative, meaning that 19 isolates were able to convert the amino acid tryptophan with the enzyme tryptopanase. Simmons citrate test resulted in 8 negative isolates, due to their inability to use citrate as a source of carbon (Reynolds 2018). The positive motility proved that all the isolated bacteria had flagella (Cowan 2004).

Table 3 presents the bacteria's ability to perform the fermentation of sugars such as glucose, lactose, maltose, mannitol, and sucrose, as an indication that bacteria produce acid, which can be seen in the color change from red to yellow (Tariq et al 2016). All bacterial isolates were able to produce gas in the glucose test. Gobbetti et al (2005) explained that bacteria can produce the enzyme hexokinase. Maltose phosphorylase is produced to reduce/ferment maltose, and only SMA isolates are unable to produce it. According to Garrity et al (2012), SN1a, SN2, SN2a, SN2b, SMb, SW1, MM3 isolates are *A. hydrophila* bacteria with morphological characteristics that were short stem, positive oxidation, positive catalase, positive motility, produce gas, fermentative, positive indole and citrate with positive results. SN, AN2, AN2a, MM2, MM, AL, Ala, Am2, AM2a, ML1, ML2a, MN2a, MN are *Plesiomonas shigelloides,* with the characteristics of gram negative, live in aquatic, flagged (motile), rod-shaped, glucose and positive oligosaccharides (Baratéla et al 2001; Pieretti et al 2008)*,* oxidase-positive (Okawa et al 2004), except SMa, which is *Pseudomonas pseudoalcaligenes* bacteria that have characteristics not related to carbon, such as sucrose and glucose and are a positive catalase (Hang et al 2002); reductive, positive in iron agar, nitrate reductions, grow well on Mac Conkey media (Davis et al 1999; Quinteira et al 2005), gram-negative bacteria, mesophilic, and does not form spores (Shi & Xia 2003).

Table 2

Bacterial isolate biochemical test results

Types of chemistry tests	*Salawati sub-district sample location*																			
	Malaus					*Majaran*		*Salawati*	*Majener*		*Mariat*		*Klamalu*		*Aimas*		*Malawili*			*Klamono*
	SN	*SN1a*	*SN2*	*SN2a*	*SN2b*	*Sma*	*SMb*	*SW1*	*AN2*	*AN2a*	*MM2*	*MM3*	*AL*	*Ala*	*Am2*	*AM2a*	*ML*	*ML2*	*ML2a*	*MN*
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidative/ fermentative test	F	F	F	F	F	NR	F	F	F	F	+	-	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	V	+	+	+	+	+	+	+	+
Simon citrate	+	-	-	-	-	V-	-	-	+	+	+	-	+	+	+	+	+	+	+	+
H2S Production	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Urease	+	-	-	-	-	V-	-	-	+	+	-	+	-	-	-	-	-	-	-	-
Indol	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	-	+	+	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-

SN, SN1a, SN2, SN2a, SN2b, Sma, SMb, SW1, AN2, AN2a, MM2, MM3, AL, Ala, Am2, AM2a, ML, ML2, ML2a, MN - code names of the cultivators; + positive; - negative; F – fermentative; NR - no reaction; V - possible +/-.

Table 3

Carbohydrate fermentation test result

Types of carbohydrate tests	*Salawati sub-district sample location*																			
	Malaus					*Majaran*		*Salawati*	*Majener*		*Mariat*		*Klamalu*		*Aimas*		*Malawili*			*Klamono*
	SN	*SN1a*	*SN2*	*SN2a*	*SN2b*	*Sma*	*SMb*	*SW1*	*AN2*	*AN2a*	*MM2*	*MM3*	*AL*	*Ala*	*Am2*	*AM2a*	*ML*	*ML2*	*ML2a*	*MN*
Glucose gas	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+
Arabinosa	-	+	+	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+
Manitol	+	V	V	V	V	-	V	V	+	+	+	V	+	+	+	+	+	+	+	+
Maltosa	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalosa	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylosa	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol/salicin	+	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+
Dulcitol	+	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+
Sucrose	-	+	+	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-

SN, SN1a, SN2, SN2a, SN2b, Sma, SMb, SW1, AN2, AN2a, MM2, MM3, AL, Ala, Am2, AM2a, ML, ML2, ML2a, MN - code names of the cultivators; + positive; - negative; F – fermentative; NR - no reaction; V - possible +/-.

There have been many explanations regarding the causes of *O. niloticus* mortality by biochemical identification of bacteria (Zheng et al 2012; Sakai et al 1993; Hamed et al 2018). The bacterial isolates obtained were selected based on morphology, gram reactions, and catalase tests (Wu et al 2012). Morphological test results of bacteria showed that all isolates were gram-negative pink, only one white colony. Gram test for bacteria is essential to know the physiological and biochemical characteristics of cells and is a fundamental character in the classification and identification of bacteria (Claus 1992). It is because the bacterial epithelium is susceptible and specific to the colors given crystal violet and safranin (Nunns et al 1997).

This study demonstrates that *O. niloticus* mortality in Sorong District was potentially caused by bacterial infections of *A. hydrophila*, *P. shigelloides*, and *P. pseudoalcaligenes*. Tables 2 and 3 show the isolates SN1a, SN2, SN2a, SN2b, SMb, SW, and MM3 were gram negative, had positive responses to oxidation, catalose, motility, gas, fermentative, indole and citrate that's showing isolates of *A. hydrophila*. The positive carbohydrate fermentation test of isolates in arabinosa, sucrose, mannitol, maltose, sucrose, and glucose was *A. hydrophila* (Janda 1985; Garrity et al 2012). SN, AN2, AN2a, MM2, MM, AL, Ala, Am2a, ML1, ML2a, MN isolates in Tables 2 and 3 had the characteristics of gram negative, positive on catalase, oxidase, OF (F), motility, lysine, ornithin, MR and indol, negative on gelatin and VP, as well as carbohydrate fermentation showed positive results in glucose, lactose, inositol, and negative in arabinose, and sucrose was *P. shigelloides* (Doyle 1989; Carter & Cole 1990). Tables 2 and 3 also show isolates that have characteristics OF (O), positive on oxidase, catalase mortality, and All carbohydrate fermentation, gelatin, lysine, arginine, ornithine all negative results, this isolate showed *P. pseudoalcaligenes* (Carter & Cole 1990). The primary cause of freshwater fish death was *A. hydrophila*, a gram-negative motile bacterium having bacilli characteristics (Fadl et al 2017). The disease caused by *A. hydrophila* bacteria called MAS is characterized by infection symptoms such as swelling of tissues, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia (Pridgeon & Klesius 2011). *P. shigelloides* are facultatively anaerobic gram-negative bacteria, with motility and oxidase-positive characteristics and are a family of Vibrionaceae that can infect fish and humans (Carter & Cole 1990). Fish infections with other *Pseudomonas* spp. bacteria, such as *Aeromonas* spp., *Vibrio* spp. and *Edwardsiella tarda* were also reported (Behera et al 2018). The bacterial genus *Pseudomonas* spp. can be found in the aquatic environment and is pathogenic also in humans (Devarajan et al 2017). *P. pseudoalcaligenes* have the following characteristics: Gram negative, oxidative (+), fermentative (+), producing carboxylic acid and are able to reduce nitrate with nitrilase (Luque-Almagro et al 2015).

Conclusions. The tested bacteria results suggest that the death cause of *O. niloticus* in Sorong district might be *A. hydrophila* and *P. shigelloides*. Failures in fish farming can be caused by infectious diseases. Detection of diseases in addition to analyzing clinical symptoms must also be accompanied by identification.

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