Received: 15 July 2020 | Revised: 25 August 2020 | Accepted: 27 August 2020

DOI: 10.1111/jfd.13270

ORIGINAL ARTICLE

Journal of Fish Diseases WILEY

Impact of a reduced water salinity on the composition of *Vibrio* spp. in recirculating aquaculture systems for Pacific white shrimp (*Litopenaeus vannamei*) and its possible risks for shrimp health and food safety

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Funding information
Parts of this study were supported by Deutsche Bundesstiftung Umwelt DBU (German Federal Environmental Foundation, Osnabrück, Germany) [Grant/ Award Number: AZ 30575-23].

Abstract

Tropical shrimp, like *Litopenaeus vannamei*, in land-based recirculating aquaculture systems (RAS) are often kept at low water salinities to reduce costs for artificial sea salt and the amount of salty wastewater. Although these shrimp are tolerant against low salinities, innate immunity suppression and changes in the microbial composition in the water can occur. As especially *Vibrio* spp. are relevant for shrimp health, alterations in the species composition of the *Vibrio* community were analysed in water from six RAS, run at 15‰ or 30‰. Additionally, pathogenicity factors including *pirA/B*, *VPI*, *toxR*, *toxS*, *vhh*, *vfh*, *tdh*, *trh*, *flagellin* genes and *T6SS1/2* of *V. parahaemolyticus* were analysed. The *Vibrio* composition differed significantly depending on water salinity. In RAS at 15‰, higher numbers of the potentially pathogenic species *V. parahaemolyticus*, *V. owensii* and *V. campbellii* were detected, and especially in *V. parahaemolyticus*, various pathogenicity factors were present. A reduced salinity may therefore pose a higher risk of disease outbreaks in shrimp RAS. Because some of the detected pathogenicity factors are relevant for human health, this might also affect food safety. In order to produce healthy shrimp as a safe food for human consumption, maintaining high water salinities seems to be recommendable.

KEYWORDS

pathogenicity factors, RAS, salinity, shrimp, *V. parahaemolyticus*

1 | INTRODUCTION

Pacific white shrimp (*Litopenaeus vannamei*) are one of the most frequently cultivated shrimp species worldwide (Ezquerra Brauer, Salazar Leyva, Bringas Alvarado, & Rouzaud Sández, 2003). Their native habitat is the eastern Pacific coast of South America with an average water temperature of above 20°C and a salinity of 34–37‰. In aquaculture, these shrimps are produced in extensive pond culture at water temperatures between 26–32°C in large quantities, mostly in South America and Asia (Bundesverband Aquakultur Garnele, 2018; FAO). By developing land-based recirculating aquaculture systems (RAS), the production of *L. vannamei* has become

Part of the data from this manuscript were already included in a doctoral thesis accepted by the University of Veterinary Medicine Hannover, Germany, on 10 June 2020.

possible also in northern European countries and North America on inland farms independent from natural sea water (Bundesverband Aquakultur Garnele, 2018; FAO, 2018). Main challenges of keeping shrimp in RAS are the maintenance of high water temperatures and appropriate salinities (Teitge, Peppler, Steinhagen, & Jung-Schroers, 2020). By using waste heat from biogas plants, the heating of RAS water can be achieved in a sustainable way. As *L. vannamei* are very tolerant against low or moderate salinity levels, RAS for on-growing shrimps after the post-larval stage are often operated at a salinity of 10–15‰ to reduce costs for artificial sea salt and the amount of salty wastewater (Bray, Lawrence, & Leungtrujillo, 1994; Jayasankar et al., 2009). This contributes further to the sustainability of a local shrimp production. However, even though shrimp tolerate brackish salinities, their innate immunity can be suppressed when the salinity in water is reduced, especially when the ion composition is suboptimal. Then, low salinity levels may lead to a higher susceptibility to bacterial pathogens like *V. alginolyticus* (Li, Yeh, & Chen, 2010; Wang & Chen, 2005). In general, disease outbreaks in shrimp aquaculture are triggered, when environmental stress increases the susceptibility of the shrimp to bacterial infections (Sung, Hsu, Chen, Ting, & Chao, 2001). Like in RAS for finfish, also in intensive shrimp aquaculture, disease outbreaks might occur due to viral, parasitic or bacterial infections (Austin & Zhang, 2006; Bauer et al., 2018; LeRoux et al., 2015; Lotz, 1997; Soto-Rodriguez, Gomez-Gil, & Lozano, 2010). By stocking the systems with specific pathogen-free post-larvae from virus-free sources, the entry of viral and parasitic pathogens can be almost completely prevented (Lotz, 1997). Nevertheless, due to high stocking densities, large amounts of organic material from faeces and non-utilized feed can accumulate, in particular in combination with low water exchange rates. Consequently, the load of heterotrophic bacteria in recirculating water can be very high in shrimp RAS and might even increase with time (Bauer et al., 2018; King et al., 2004; Schreier, Mirzoyan, & Saito, 2010). In marine and brackish water RAS, a great variety of *Vibrio* spp. can be found. *Vibrio* spp. are ubiquitous bacteria in sea water and brackish water, with a share of the bacterial community of up to 40% (Buller, 2014; Urakawa & Rivera, 2006). Several *Vibrio* spp. form a part of the natural microflora of fish and shellfish (Otta, Karunasagar, & Karunasagar, 1999; Ruangpan & Kitao, 1991; Vanderzant, Nickelson, & Judkins, 1971), but some species can also act as facultative pathogens for shrimp or finfish. In particular, *V. alginolyticus*, *V. campbellii*, *V. harveyi*, *V. owensii* and *V. parahaemolyticus* have already been identified as potential pathogens for shrimp (Karunasagar, Pai, Malathi, & Karunasagar, 1994; Li, Wang, & Sun, 2011; Li et al., 2017; Liu, Cheng, Hsu, & Chen, 2004; Liu et al., 2015, 2017, 2018; Xiao et al., 2017). Some species of *Vibrio* are known to be potentially pathogenic for humans as well, and especially, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are major causes of seafood-borne illnesses (Fernando, Krishnan, Fairweather, & Ericsson, 2011; Gopal et al., 2005; Haendiges et al., 2014; Hwang et al., 2000; Jeon, Lee, & Song, 2009; Jung, 2018; Kim, Park, Kil, Lee, & Suh, 2000; Kothary, Lowman, McCardell, & Tall, 2003; Letchumanan, Chan, & Lee, 2014; Liu et al., 2018; McLaughlin et al., 2005; Nelapati & Chinnam, 2012; Park et al., 1997; Son et al., 1998; Su & Liu, 2007; Wang et al., 2015; Yeung & Boor, 2004). Seafood-associated disease outbreaks in humans have been reported worldwide (Daniels et al., 2000; Hoi, Larsen, Dalsgaard, & Dalsgaard, 1998; do Nascimento, Vieira, Theophilo, Rodrigues, & Vieira, 2001), and depending on the *Vibrio* species involved, the clinical manifestations range from gastroenteritis to septicaemia and wound infection (Farmer & Hickman-Brenner, 1992; Oliver & Kaper, 1997; Ulusarac & Carter, 2004). Potentially pathogenic *Vibrio* spp. for humans cannot only be found in natural habitats or pond aquaculture systems, but also in RAS (Bauer et al., 2018).

Alterations in the microbiological community in RAS water can be induced by changing culture conditions, including temperature, stocking density, feeding regimes and salinity and might have negative effects for the animals kept in the systems. To safeguard animal health, previous studies point towards the importance of a controllable and stable bacterial diversity in the rearing water of RAS by using "matured" intake water (Attramadal et al., 2014, 2016; Skjermo, Salvesen, Oie, Olsen, & Vadstein, 1997). In order to assess the risk of disease outbreaks in shrimp and of food contaminations originating from *Vibrio* spp. present in a RAS, specific virulence genes have been used as molecular markers for potential pathogens. Due to its relevance to human health, *V. cholerae* is the most extensively investigated species in the genus *Vibrio*. Its virulence is determined by many factors such as activators for virulence genes (*toxR*, *toxS*) that encode, for example cholera toxin (*ctx*) production. *ToxR* is often expressed in *V. parahaemolyticus* as well as in other *Vibrio* spp. and is involved in their regulatory functions (Kim et al., 1999; Pang et al., 2006). The *Vibrio cholerae* pathogenicity island (*VPI*) contains various genes that code for virulence factors, for example the toxin co-regulated pilus (*TCP*), which enhances bacterial colonization of intestinal mucosa (DiRita, Parsot, Jander, & Mekalanos, 1991; Miller, DiRita, & Mekalanos, 1989; Sechi, Dupre, Deriu, Fadda, & Zanetti, 2000). Factors that were found to be relevant for the virulence s of *V. parahaemolyticus* are the type VI secretion systems (*T6SS1* and *T6SS2*). *T6SS1* can improve the growth and distribution of *V. parahaemolyticus* due to toxic effects on other bacteria, especially in warm and high salinity environments, whereas *T6SS2* is active in a low salinity environments (Salomon, Gonzalez, Updegraff, & Orth, 2013). One of the most important virulence factors of bacteria is flagella that are essential for motility (Thompson, Austin, & Swings, 2006). Nonetheless, other virulence factors (e.g. toxin production) can limit overreactions of immune responses in order to increase bacterial survival (Hornef, Wick, Rhen, & Normark, 2002). Additional factors that increase the virulence of *Vibrio* spp. are haemolysins that destroy the cell membrane of erythrocytes and induce haemorrhagic septicaemia, bloody gastroenteritis and diarrhoea (Ichinose et al., 1987). Many *Vibrio* spp. are known to carry genes that encode for haemolysins, such as *V. parahaemolyticus*, *V. harveyi* or *V. fluvialis* (Bai et al., 2008; Bej et al., 1999; Han et al., 2002). In recent years, also specific pathogenicity factors for shrimp were examined. Some of the most important pathogenicity factors in *Vibrio* spp. are the *pirAB* toxin genes located on an extrachromosomal plasmid that are encoding the binary toxins *V. parahaemolyticus Photorhabdus* insect-related

toxins ($PirA^{Vp}$ and $PirB^{Vp}$). These genes could be first detected in isolates of *V. parahaemolyticus* but meanwhile also in *V. owensii* and *V. campbellii* isolates (Dong et al., 2017; Liu et al., 2015, 2017, 2018; Xiao et al., 2017). The induced disease is known as acute hepatopancreatic necrosis disease (AHPND), and affected shrimp show massive sloughing of tubule epithelial cells of the hepatopancreas (Han, Tang, Tran, & Lightner, 2015; Sirikharin et al., 2015). Disease outbreaks due to AHPND with mortalities up to 100% occur in *L. vannamei* and *P. monodon* and are reported from South-East Asia and Mexico (de la Pena et al., 2015; Han et al., 2015; Joshi et al., 2013; Lee et al., 2015; Lightner, Redman, Pantoja, Noble, & Tran, 2012; Nunan, Lightner, Pantoja, & Gomez-Jimenez, 2014; Soto-Rodriguez, Gomez-Gil, Lozano-Olvera, Betancourt-Lozano, & Morales-Covarrubias, 2015; Thitamadee et al., 2016; Tran et al., 2013).

To investigate the influence of a lowered salinity in shrimp RAS on the composition of *Vibrio* spp., in the present study, the *Vibrio* spp. in water of shrimp RAS of salinities of 15 and 30‰ were compared. Additionally, the pathogenicity factors of the main detected species at each salinity were analysed to assess the potential risk for shrimp health and food safety.

2 | MATERIAL AND METHODS

2.1 | RAS

Water samples originated from six different RAS stocked with *L. vannamei* of different ages and under different production conditions (Table 1). Three RAS were maintained at a salinity of around 30‰, and three RAS were maintained at a salinity of around 15‰. RAS 1, 3, 5 and 6 were located in different research facilities. RAS 1 and RAS 3 consisted of three 100-L volume holding tanks. RAS 5 and RAS 6 consisted of three 70 L volume holding tanks. All RAS in research facilities were stocked with specific pathogen-free *L. vannamei* from a hatchery (Shrimp improvement systems, Singapore 718873, Facility Florida). Samples from RAS 2 and RAS 4 originated from commercial production facilities for *L. vannamei* both located in Northern Europe. RAS 2 consisted of two 7,000 L volume holding tanks, and RAS 4 consisted of four 88,000 L volume holding tanks. Water samples from each holding tank of both RAS were analysed.

2.2 | Sampling and analyses for total bacterial amount

From each tank of each RAS, water samples of 100 ml were analysed. The number of samples that were investigated per tank is given in Table 1. For analysing the total amount of bacteria, the number of colony-forming units (cfu) per ml water was determined. For this, all water samples were serially diluted with a sterile 2% sodium chloride solution. From samples of undiluted water to a dilution level of 10^{-5}, 100 µl of each were plated out in duplicate on Columbia Sheep Blood (CSB) agar and Columbia Sheep Blood agar with 2% sodium chloride, and incubated at 25°C for 48 hr. Colony-forming units (Cfu) on the plates were counted after 12 and 48 hr, and the amount of Cfu per mL of tank water was calculated. The amount of morphologically different Cfus was assessed semi-quantitatively (low: +; up to ten colonies/ plate, moderate: ++; 10–50 colonies/ plate), high: +++; >50 colonies/plate), and all morphologically different colonies were sub-cultured on sheep blood agar with 2% sodium chloride and on Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar (Oxoid Deutschland GmbH, Germany). After a second 48-hr incubation period at 25°C, subcultures were stored at −80°C in 1 ml of veal infusion broth until further analysis for identification of the bacterial species. All *V. parahaemolyticus* isolates (identified by *pyrH* sequencing, as described below) were additionally sub-cultivated on CSB agar and on CSB agar with 2% sodium chloride at 37°C for 24 hr.

2.3 | Identification of *Vibrio* species at different salinities

Vibrio spp. were identified either by sequencing a fragment of the uridylate kinase encoding gene *pyrH* or by sequencing a fragment of the 16S rRNA gene because both methods proved to be very reliable for identifying *Vibrio* spp. from shrimp aquaculture (Bauer et al., 2018). For this, DNA was extracted from single colonies of each isolate using a commercially available DNA extraction kit in accordance with the manufacturer´s instructions (Qiagen GmbH). DNA concentration was measured using spectrophotometry (NanoDrop ND-1000 Lab, PeqLab Biotechnologie GmbH), and the DNA content was adjusted using PCR grade water (Thermo Fisher Scientific Inc.) to a concentration of 10 ng/µl. For analysing the *pyrH* gene, an

TABLE 1 Characteristics (RAS category, shrimp age, water salinity and temperature, no. of tanks per RAS sampled and no. of samples per tank) of the six examined RAS

RAS no.	System	Shrimp age	Salinity (‰)	Water temp. (°C)	No. of tanks sampled	No. of samples per tank
1	Laboratory	Sub-adult	30	30	3	4
2	Commercial	Post-larvae	28.5	28.7	2	1
3	Laboratory	Post-larvae	30	30	3	5
4	Commercial	Adult	15.5	28.5	4	1
5	Laboratory	Post-larvae	15	30	4	4
6	laboratory	Sub-adult	15	30	4	4

endpoint PCR was performed with 0.25 U of hot-start KAPA 2G robust polymerase (PeqLab Biotechnologie GmbH), 5× KAPA2G A buffer, 200 nM of each primer (Thompson et al., 2005), 200 µM of each dNTP, 5.0 µl of DNA samples and nuclease-free water to a final volume of 25 µl. The V1-V9 region of the 16 S rRNA-encoding gene was amplified using forward and reverse primers designed by Jiang et al. (Jiang, Gao, Xu, Ye, & Zhou, 2011). An endpoint PCR was performed with 0.2 U of hot-start KAPA 2G robust polymerase (PeqLab Biotechnologie GmbH), 1× KAPA A buffer, 200 nM of each primer, 200 µM of each dNTP, 5.0 µl of DNA samples and nuclease-free water to a final volume of 25 µl.

PCRs were performed in a SensoQuest thermocycler (SensoQuest GmbH) with a PCR profile consisting of an initial denaturation step at 95°C for 5 min, five cycles at 95°C for 30 s, 63–58°C for 30 s, 72°C for 60 s (every cycle with annealing temperature 1°C lower), followed by 35 cycles at 95°C for 30 s, 57°C for 30 s, 72°C for 60 s, and an extension step at 72°C for 7 min. Sequencing of PCR products was performed by LCG Genomics GmbH, Berlin, Germany. The obtained sequences were compared to known sequences using the Standard nucleotide BLAST database (National Center for Biotechnology Information, U.S. National Library of Medicine, https://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparing the *pyrH* sequences, and the online database EzBioCloud (http://www.ezbiocloud.net) for comparing the 16S rRNA sequences. The results were analysed for species composition in the six RAS, and especially for differences in the species composition at different salinities.

2.4 | Analysis of pathogenicity factors of *Vibrio* spp

A representative number of isolates from the most abundant species of the identified *Vibrio* spp. were analysed for pathogenicity factors. Isolates from the following species were included in the analyses: *V. alginolyticus* ($n = 10$), *V. campbellii* ($n = 8$), *V. fluvialis* ($n = 5$), *V. harveyi* ($n = 13$), *V. navarrensis* ($n = 6$), *V. owensii* ($n = 14$), *V. parahaemolyticus* ($n = 11$), *V. pelagius* ($n = 2$), *V. rotiferianus* ($n = 6$), *V. tubiashii* ($n = 7$) and *V. xuii* ($n = 6$). As standards, eight type strains of *Vibrio* spp. from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) were included in the analyses: *V. alginolyticus* (DSM 2171; ATCC 17749), *V. fluvialis* (DSM 10283; ATCC 33809), *V. harveyi* (DSM 19623; ATCC 14126), *V. parahaemolyticus* (DSM 10027; ATCC 17802), *V. pelagius* (DSM 21205; ATCC 25916), *V. rotiferianus* (DSM 17186), *V. tubiashii* (DSM 19142; ATCC 19109) and *V. vulnificus* (DSM 10143; ATCC 27562). Examined pathogenicity factors were *pirA* and *pirB* of *V. parahaemolyticus* (Wangman et al., 2018), flagellin B and C of *V. harveyi* (Bai et al., 2008), *toxR* (Gomez-Gil et al., 2004), *toxS* and the *Vibrio cholerae* pathogenicity island (*VPI*) of *V. cholerae* (Sechi et al., 2000), the thermostable direct haemolysin (*tdh*) and the tdh-related haemolysin (*trh*) of *V. parahaemolyticus* (Bej et al., 1999), the *Vibrio harveyi* haemolysin (*vhh*) (Ruwandeepika, Sanjeewa Prasad Jayaweera, Paban Bhowmick, Karunasager, Bossier, & Defoirdt, 1988; Wang, Zhang, Zhong, Sun, & Chen, 2007), the *Vibrio fluvialis* haemolysin (*vfh*) (Han et al., 2002), the flagellins B, C, D of *V. vulnificus* (Vandenberghe et al., 1999) and the type six secretion systems 1 and 2 (*T6SS1*, *T6SS2*) of *V. parahaemolyticus* (Salomon et al., 2013). For each pathogenicity factor, a gene fragment was amplified using the forward and reverse primers and the respective PCR protocol as detailed in Table 2. PCR products were applied to a 1% agarose gel with the addition of 4 µl Gel Red Nucleic Acid DNA marker (Biotium, Inc.) and 1 x TBE (Tris-Boric Acid-EDTA) buffer. DNA amplicons were subsequently separated in an electrical field. A DNA ladder (100 bp, Carl Roth GmbH) was used to determine the product size. The resulting bands were visualized under 302 nm UV light. PCR products from all isolates, where a band of the appropriate molecular size had been obtained after electrophoresis, were sequenced by LGC Genomics GmbH.

Isolates showing aberrant bands or multiple bands were sequenced as well in order to verify the result or exclude false-positive samples. All obtained sequences were compared with sequences found in the Standard nucleotide BLAST database (National Center for Biotechnology Information, U.S. National Library of Medicine, https://blast.ncbi.nlm.nih.gov/Blast.cgi). All obtained sequences were submitted to the BankIt database (Table S1).

2.5 | Analysis of motility

Motility was observed microscopically by means of the hanging drop method (Buller, 2014) in 96 *Vibrio* isolates including the type strains. The level of motility was determined using the following scheme:

0 = no motility of bacteria (passive drifting or Brownian movement)
1 = sedentary movement of bacteria
2 = sedentary movement and less than 20% of the bacteria showing forward movement
3 = sedentary movement and 20%–50% of the bacteria showing forward movement
4 = sedentary movement and 50%–100% of bacteria showing forward movement

2.6 | Analysis of haemolysis

All cultures showing either α- or β-haemolysis on CSB agar with and without the addition of 2% sodium chloride for 48 hr at 25°C were rated positive.

2.7 | Statistical analysis

The data of the total amounts of bacteria in the water samples were statistically analysed using the computer program SigmaPlot 12 by an ANOVA on ranks, followed by an all-pairwise multiple comparison procedure. Differences between tested data sets were considered significant at a probability of error of $p < .05$. Principal component

TABLE 2 Primers and PCR protocols of the analysed toxins PirA and PirB, flagellins, haemolysins and of pathogenicity factors of *V. cholerae* and the type six secretion systems 1 and 2 of *V. parahaemolyticus*

	Gene	Primer sequence (5′–3′)	Product size (bp)	Reference	PCR protocol
Toxins $PirA^{Vp}$/ $PirB^{Vp}$	*Photorhabdus* insect-related toxins ($PirA^{Vp}$ and $PirB^{Vp}$)	f: GTGTTGCATAATTTTGTGCA r: GTCATGTTCGATATTGAAGC	1762	Wangman et al., 2018	1:94°C; 2 min 2:94°C, 30 s 3:56°C; 30 s 4:68°C, 2 min (30×) 5:68°C, 10 min
Flagellins	Flagellin B (*flaB*) of *V. harveyi*	f: AACGTATCAGCGATGACC r: TTGAAACGGTTCTGGAAT	928	Bai et al., 2008	1:94°C; 3 min 2:94°C, 1 min 3:56°C; 30 s 4:72°C, 30 s (30×) 5:72°C, 5 min
	Flagellin C (*flaC*) of *V. harveyi*	f: AAATCATTCCAAATCGGTGC r: TCTTTGATTCGGCTCTTA	580		1:95°C; 5 min 2:95°C, 30 s 3:55°C; 15 s 4:72°C, 15 s (33×) 5:72°C, 5 min
	Flagellin B (*flaB*) of *V. vulnificus*	f:CGTAGACAGCCACCGTGCAGAGC r: GAGATTTCTGTCTAGTTAAGGC	Not indicated in the reference	Kim et al., 2014	1:95°C; 5 min 2:95°C, 30 s 3:55°C; 15 s 4:72°C, 15 s (33×) 5:72°C, 5 min
	Flagellin C (*flaC*) of *V. vulnificus*	f: ATCGGTTCGTCACTCAAACC r: ATTGCCAGAGATGGGTGAAG	Not indicated in the reference		1:95°C; 5 min 2:94°C, 30 s 3:54°C; 30 s 4:72°C, 30 s (25×) 5:72°C, 7 min
	Flagellin D (*flaD*) of *V. vulnificus*	f: TTAACCGCTGCTGCTCAGAG r: AGAACCGTTTCAACCATGC	Not indicated in the reference		See Flagellin C of *V. vulnificus*

(Continues)

TABLE 2 (Continued)

	Gene	Primer sequence (5′–3′)	Product size (bp)	Reference	PCR protocol
Haemolysins	*V. harveyi* haemolysin	f: TTCACGCTTGATGGCTACTG r: GTCACCCAATGCTACGACCT	234	Ruwandeepika et al., 2010	1:95°C; 5 min 2:95°C, 30 s 3:58°C; 30 s 4:72°C, 30 s (30×) 5:72°C, 5 min
		f: CACTTATGTCCGCTGCTGGT r: GCTGTGGTCGGGTGTGTAC	1,058	Wang et al., 2007	
	V. fluvialis haemolysin	f: GACCGAACATAAAGGGGAAC r: GCGGAAATAGGCATCCAAC	460	Han et al., 2002	1:95°C; 5 min 2:94°C, 30 s 3:54°C; 30 s 4:72°C, 30 s (25×) 5:72°C, 7 min
	thermostable direct haemolysin (*tdh*) of *V. parahaemolyticus*	f:GTAAAGGTCTCTGACTTTTGGAC r:TGGAATAGAACCTTCATCTTCACC	269	Salomon et al., 2013	1:95°C; 5 min 2:95°C, 1 min 3:62°C; 1 min 4:72°C, 1 min (35×) 5:72°C, 2 min
	tdh-related haemolysin (*trh*) of *V. parahaemolyticus*	f: TTGGCTTCGATATTTTCAGTATCT r:CATAACAAACATATGCCCATTTCCG	500		See *tdh*
Pathogenicity factors of *V. cholerae*	*toxR*	f: GATTAGGAAGCAACGAAAG r: GCAATCACTTCCACTGGTAAC	658	Gomez-Gil et al., 2004	1:94°C; 3 min 2:94°C, 1 min 3:55°C; 1 min 4:72°C, 1 min (30×)
	toxS	f: CCACTGGCGGACAAAATAACC r: AACAGTACCGTAGAACCGTGA	640	Sechi et al., 2000	see toxR 3:56°C; 1 min
	V. cholerae pathogenicity island (*VPI*)	f: GCAATTTAGGGGCGCGACGT r: CCGCTCTTTCTTGATCTGGTAG	680		1:94°C; 5 min 2:94°C, 30 s 3:63°C; 15 s 4:72°C, 20 s (30×) 5:72°C, 5 min
Type six secretion systems	T6SS1 of *V. parahaemolyticus*	f: CACGTGACGGCTCGGTGG r: CTCTTCTTTCGCGTCTTGGTCG	Not indicated in the reference	Salomon et al., 2013	1:94°C; 3 min 2:94°C, 1 min 3:68°C; 30 s 4:72°C, 30 s (30×) 5:72°C, 5 min
	T6SS2 of *V. parahaemolyticus*	f: CGAGTATCCACTCGAAACTTTC r: TTCTGCTCCCTCAGTACTTTCTG	Not indicated in the reference		See T6SS1 3:61°C; 30 s

Note: 1 = initial denaturation; 2 – 4 = cycles (denaturation, annealing, extension); 5 = final extension

TABLE 3 Total bacterial amount (cfu/mL) in RAS run at 15 and 30 ‰

	Total bacterial amount in RAS water (cfu/ml)				
	Tank 1	Tank 2	Tank 3	Tank 4	Mean value of all tanks sampled
30‰					
RAS 1	$9.80 \times 10^5 \pm 1.27 \times 10^6$	$9.51 \times 10^5 \pm 1.10 \times 10^6$	$2.45 \times 10^5 \pm 4.82 \times 10^5$	Not sampled	$7.25 \times 10^5 \pm 4.16 \times 10^5$
RAS 2	5.18×10^6	3.15×10^5	Not sampled	Not sampled	$2.75 \times 10^6 \pm 3.44 \times 10^6$
RAS 3	$5.24 \times 10^5 \pm 1.04 \times 10^6$	$4.66 \times 10^5 \pm 8.59 \times 10^5$	$4.61 \times 10^5 \pm 7.87 \times 10^5$	Not sampled	$4.84 \times 10^5 \pm 3.53 \times 10^4$
Mean 30‰	$1.32 \times 10^6 \pm 1.24 \times 10^6$				
15‰					
RAS 4	8.60×10^4	8.30×10^4	5.20×10^4	7.30×10^6	$1.88 \times 10^6 \pm 3.61 \times 10^6$
RAS 5	$2.23 \times 10^4 \pm 6.02 \times 10^3$	$1.66 \times 10^4 \pm 7.56 \times 10^3$	$7.10 \times 10^4 \pm 3.73 \times 10^4$	$2.14 \times 10^4 \pm 1.52 \times 10^4$	$3.28 \times 10^4 \pm 2.56 \times 10^4$
RAS 6	$8.05 \times 10^4 \pm 5.77 \times 10^3$	$3.05 \times 10^4 \pm 1.11 \times 10^4$	$2.65 \times 10^4 \pm 7.00 \times 10^3$	$1.22 \times 10^5 \pm 9.50 \times 10^4$	$6.61 \times 10^4 \pm 4.60 \times 10^4$
Mean 15‰	$6.60 \times 10^5 \pm 1.06 \times 10^6$				

Note: Shown are the mean values and standard deviations of water samples from the different tanks analysed per individual RAS (number of examined tanks and samples per tank are given in Table 1), and the calculated mean values and standard deviations of the water samples from RAS run at different salinities.

analysis (PCA) was performed using the Excel Add-in Analyse Function for examining the composition of *Vibrio* species at different salinities.

3 | RESULTS

3.1 | Total bacterial amount in RAS water

The total bacterial amount was slightly higher in the RAS run at 30‰ than in the RAS run at 15‰ (Table 3). Slight differences also occurred between the commercial RAS and the laboratory RAS, whereas the number of Cfus was in general lower in the laboratory RAS (Table 3). However, no statistically significant differences were seen between the samples of the different RAS, as there were high variances between the different tanks tested in each RAS.

3.2 | Composition of *Vibrio* spp. in RAS water

In total, 321 isolates of *Vibrio* spp. were detected in the water samples of the six RAS (Table 4). From these isolates, 269 could be identified at species level and among these, 18 different *Vibrio* species were identified. For 52 isolates, an explicit identification at species level was not possible, either by 16S rRNA or by *pyrH* sequencing.

Vibrio alginolyticus and *V. harveyi* were the most prevalent species in all examined RAS, whereas *V. alginolyticus* was detected almost twice as often in the RAS run at 30‰ than at 15‰. Large differences occurred in the prevalence of other Vibrio species dependent on the salinity of the water. In the RAS run at 30‰, *V. rotiferianus* and *V. tubiashii* were detected in high numbers but were not present or only detectable to a much lesser abundance in the RAS run at 15 ‰. In the RAS run at a salinity of 15‰, on the other hand, high numbers of *V. campbelli*, *V. owensii* and especially *V. parahaemolyticus* could be detected, which were virtually absent in the RAS with a salinity of 30‰.

A semiquantitative evaluation of the amounts of the isolated *Vibrio* species resulted in a different proportional composition of the *Vibrio* community in the RAS run at different salinities (Figure 1). *Vibrio alginolyticus* represented between 21% and 47% of the percentage proportion of the total *Vibrio* amount in RAS with a salinity of 30‰. However, this bacterium was not detected in one of the RAS with a salinity of 15‰ and represented 11 and 37% of the total percentual bacterial amount in the two other RAS of this salinity. In addition, the percentual amounts of *V. chagasii*, *V. pectenicida*, *V. tubiashii* and *V. xuii* were higher in the RAS at 30‰ compared with the RAS run at a salinity of 15‰. A strikingly higher percentual amount of *V. parahaemolyticus*, *V. owensii* and *V. campbellii* was present in the RAS run at a salinity of 15‰. *Vibrio parahaemolyticus* showed a mean percentual amount in the RAS at 15‰ of 16.5%, whereas in the RAS at 30‰, the amount of the bacterium was only 0.4%. *Vibrio owensii* could not be detected at

TABLE 4 Number of different *Vibrio* species isolated from the six examined RAS

Species	All RAS	All RAS at 30 ‰	RAS 1	RAS 2	RAS 3	All RAS at 15 ‰	RAS 4	RAS 5	RAS 6
V. alginolyticus	62	39	25	5	9	23	0	7	16
V. campbellii	10	0	0	0	0	10	8	2	0
V. chagasii	1	1	0	1	0	0	0	0	0
V. cidicii	3	0	0	0	0	3	3	0	0
V. fluvialis	5	0	0	0	0	5	5	0	0
V. fortis	3	1	0	0	1	2	0	0	2
V. harveyi	60	30	22	0	8	30	9	19	2
V. hepatarius	2	2	0	0	2	0	0	0	0
V. mytili	3	1	0	0	1	2	0	2	0
V. navarrensis	7	0	0	0	0	7	7	0	0
V. orientalis	1	0	0	0	0	1	0	1	0
V. owensii	12	0	0	0	0	12	12	0	0
V. parahaemolyticus	36	1	0	0	1	35	11	10	14
V. pectenicida	2	2	0	0	2	0	0	0	0
V. pelagius	15	7	4	1	2	8	0	0	8
V. plantisponsor	2	0	0	0	0	2	2	0	0
V. rotiferianus	26	19	16	0	3	7	4	3	0
V. tubiashii	13	13	13	0	0	0	0	0	0
V. xuii	6	6	1	2	3	0	0	0	0
Vibrio spp.	52	25	19	0	6	27	4	14	9

Note: Shown are the total numbers for all RAS, the numbers of individual species detected in the RAS with a water salinity of 15 and 30 ‰ and the number of individual species detected in each of the six investigated RAS.

all in the RAS run at a salinity of 30‰ but was present at a mean percentual amount of 20% in the RAS run at a salinity of 15‰. In the RAS run at 15‰, the percentual amounts of *V. cidicii*, *V. fluvialis*, *V. navarrensis* and *V. plantisponsor* were also higher compared to the RAS run at 30‰.

Within the three RAS operated at the same water salinity, a very similar bacterial composition was found, and in a PCA analysis, two clearly defined clusters were seen (Figure 2).

3.3 | Analysis of pathogenicity factors

In total, 96 of the 321 *Vibrio* isolates from the water samples of RAS and eight reference strains were analysed for the presence of genes encoding pathogenicity factors. The isolates were exemplary selected from different species and different RAS systems including laboratory scale RAS and commercial RAS.

None of the examined isolates were tested positive for *pirAB*. Analyses of motility revealed that most examined *Vibrio* isolates were motile. Bacterial movement was detected in 78 of the analysed 88 isolates. Within the same species, motile and non-motile isolates were detected and also the level of motility varied between different isolates of the same species. The detection of specific flagellin gene segments within the genome of the isolates did not correlate with the observed motility in all cases. For example, although all *V. parahaemolyticus* isolates carried the *flaB* gene, one isolate was found to be immotile in the motility test. Most *V. navarrensis* isolates were highly motile, yet none of the tested genes encoding flagellins could be detected.

The flagellin genes B and C of *V. harveyi* could be amplified only from a few *V. harveyi* isolates. However, other analysed *Vibrio* spp., namely *V. alginolyticus*, *V. fluvialis*, *V. owensii*, *V. parahaemolyticus*, *V. pelagius*, *V. rotiferianus*, *V. tubiashii* and *V. xuii*, were also found to carry these genes.

Either α- or β-haemolysis on CSB agar or CSB agar with 2% sodium chloride was detected in 42 isolates. PCR analysis showed that only 15 *Vibrio* spp. isolates carried any of the analysed haemolysin genes (Table S1). The gene encoding the *V. fluvialis* haemolysin was detected in all isolates of *V. fluvialis*, although one of these *V. fluvialis* isolates did not show haemolysis on blood agar plates. The *V. fluvialis* haemolysin gene, however, was not detected in any other species. The *V. harveyi* haemolysin gene was detected in seven of 14 *V. harveyi* isolates, including the respective type strain DSM 19623. The *V. harveyi* haemolysin gene was also detected in one *V. pelagius* and one *V. xuii* isolate. All tested isolates and type strains were negative for the haemolysin genes *tdh* and *trh* from *V. parahaemolyticus*, including all *V. parahaemolyticus* isolates. Likewise, the gene encoding the thermostable haemolysin *tdh* was not detected in any of the *V. parahaemolyticus* isolates cultivated at 37°C.

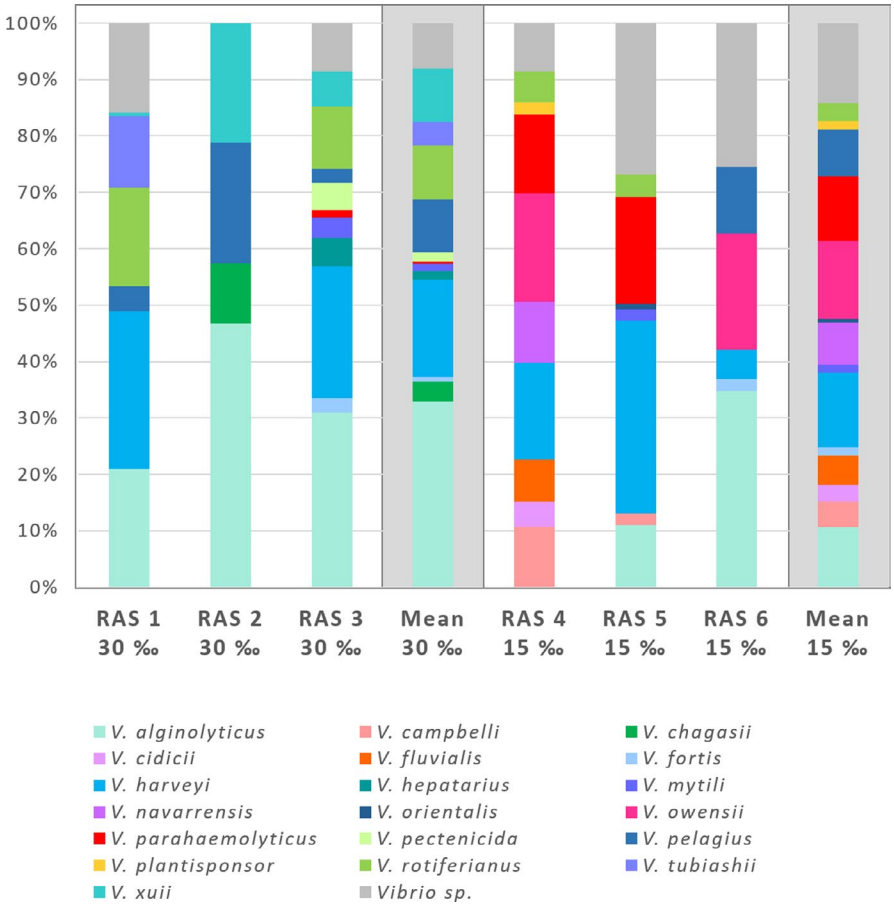

FIGURE 1 Composition of the *Vibrio* species community in the tank water of six RAS stocked with *L. vannamei*. Three RAS were run at a salinity of around 30‰, and three RAS were run at a salinity of around 15‰. Shown are the compositions for every individual RAS as well as the mean composition of *Vibrio* species for the three RAS run at the same water salinity [Colour figure can be viewed at wileyonlinelibrary.com]

All *V. parahaemolyticus* isolates, including the type strain, were analysed for the presence of the gene encoding the type VI secretion systems 1 and 2. In the culture with no salt (CSB) as well as in the saline culture (CSB with 2% sodium chloride), all isolates from water samples were found negative for the gene encoding T6SS1. However, the type strain DSM 10027 was found positive for this gene in both cultures. In contrast, sequences specific for the gene encoding T6SS2 could be found in all tested isolates (Table 5, Table S1). None of the tested isolates carried all three of the pathogenicity factors *VPI*, *toxR* or *toxS* (Table S1). *VPI* was detected in eight isolates including two *V. harveyi*, four *V. rotiferianus* and two *V. owensii* isolates. None of these isolates carried either *toxR* or *toxS*. Eighteen isolates, seven *V. alginolyticus*, eight *V. parahaemolyticus* isolates, their respective type strains DSM 2171, DSM 10027 and the type strain of *V. fluvialis* DSM 19283 carried both *toxR* and *toxS*. In three *V. fluvialis* isolates, three *V. owensii* isolates and in one *V. xuii* isolate, only *toxS* was detected. In two *V. alginolyticus*, three *V. parahaemolyticus*, one *V. owensii* and in one *V. pelagius* isolate, only *toxR* was detected.

4 | DISCUSSION

In recent years, tropical shrimp, like *Litopenaeus vannamei*, are more and more frequently produced in Northern European countries in land-based recirculation systems far away from the sea. The maintenance of an optimal chemical and microbiological water quality is crucial for shrimp health in RAS, especially when only low amounts of water are exchanged during the production cycle. It could be shown that issues in water temperature and chemical water quality, like increased ammonia and nitrite concentrations as well as suboptimal pH levels, might lead to a higher susceptibility of the animals to bacterial infections, for example, caused by *Vibrio alginolyticus* (Cheng, Wang, & Chen, 2005; Li & Chen, 2008; Liu & Chen, 2004; Tseng & Chen, 2004). Changes in water salinity are crucial for shrimp health as well. Although the isosmotic points of *L. vannamei* are at 24‰ (Castille & Lawrence, 1981), they are capable of withstanding salinities of 4 up to 45‰ (Ponce-Palafox, Martinez-Palacios, & Ross, 1997; Roy, Davis, Saoud, & Henry, 2007). This is exploited in RAS for tropical shrimp, and often the salinity is reduced to 10–15‰ after the post-larval stage to reduce the costs for artificial sea salt, and also to lower the salt content in wastewater (Bray et al., 1994; Jayasankar et al., 2009). When culturing *L. vannamei* in high salinities of 25 up to 45‰, they maintain good growth (Ponce-Palafox et al., 1997), but keeping them at low salinities requires the maintenance of optimal potassium (K^+) and magnesium (Mg^{2+}) concentrations in the artificial sea water to achieve optimal weight gains (Roy et al., 2007). Furthermore, also the ratios of Na: K (sodium: potassium) and Mg: Ca (magnesium: calcium) have to be considered as possible disruptive factors and need to be kept within optimal ranges (Roy et al., 2007). If these ions are not adjusted in shrimp aquaculture, low salinity may lead to a higher susceptibility to bacterial

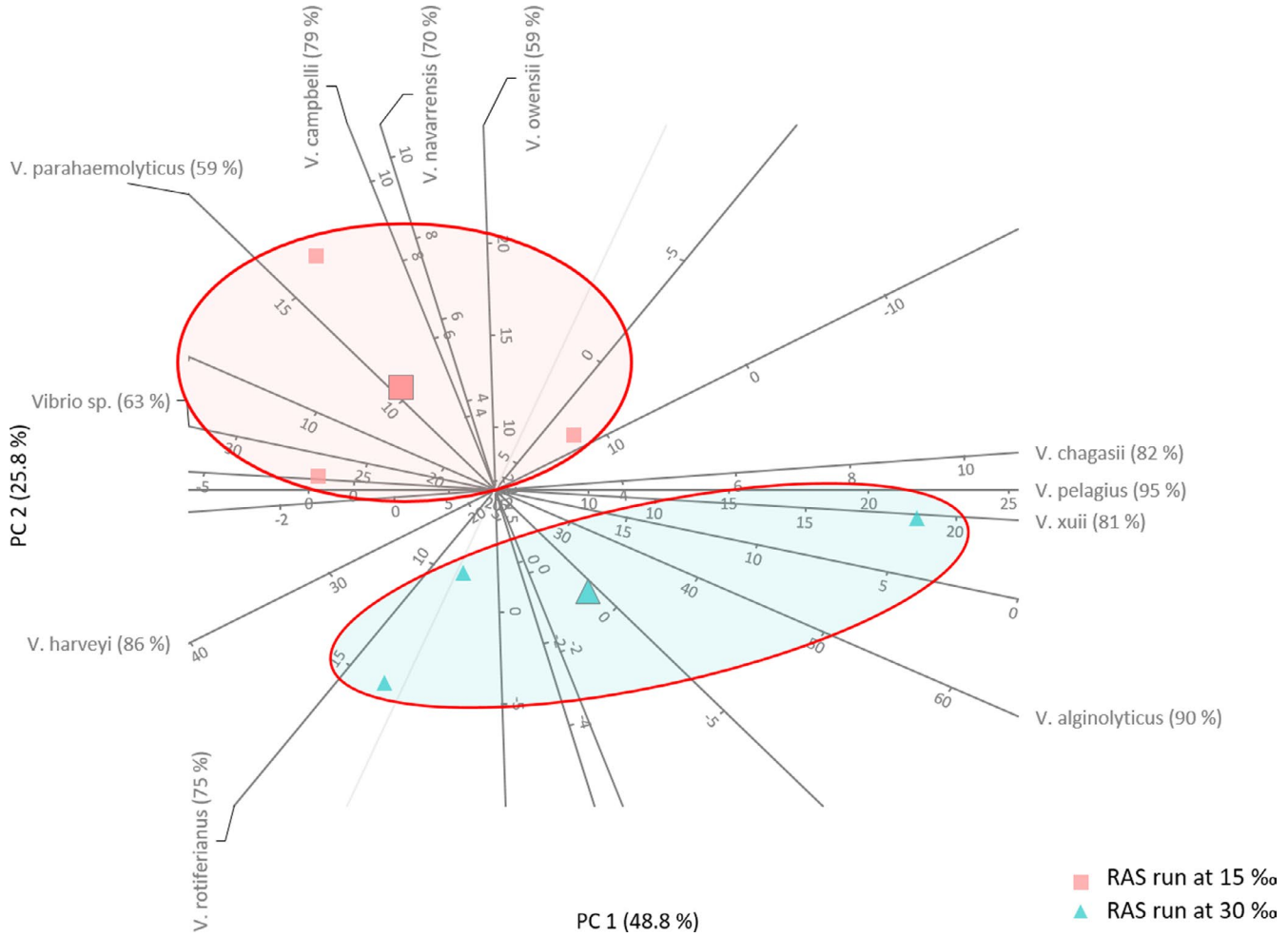

FIGURE 2 Principal component analysis (PCA) of the composition of *Vibrio* species in the tank water of six RAS stocked with *L. vannamei*. Three RAS were run at a salinity of around 30‰, and three RAS were run at a salinity of around 15‰. The data from the individual RAS are indicated with small symbols, and the mean values of the samples from RAS run at the same water salinity are indicated in large, bordered symbols. To demonstrate the differences in the *Vibrio* composition in water samples of different salinities, all samples taken from 15‰ RAS and all samples taken from 30‰ RAS are encircled together [Colour figure can be viewed at wileyonlinelibrary.com]

pathogens like *V. alginolyticus* (Wang & Chen, 2005). After larvae of *Penaeus monodon* were experimentally infected with luminous bacteria closely related to *Photobacterium* and *Vibrio*, significantly higher mortalities were seen at low salinities of 10 and 15‰ compared to higher salinities (Prayitno & Latchford, 1995). For *P. monodon* also a higher susceptibility to White spot syndrome virus at salinities below 15‰ was seen (Tendencia & Verreth, 2011). In brackish water ponds for *P. monodon*, the counts of total heterotrophs, presumptive *Vibrio* spp. and luminous *Vibrio* spp. increased with increased salinity and were highest at a salinity of 15–26‰, but the culture period of the shrimp had to be extended because of slower growth by about 12–18 days in low salinities (4–9‰) and even by about 20–30 days at moderate salinities (9–15‰) (Abraham & Sasmal, 2009). The authors therefore concluded that despite higher counts of heterotrophs and *Vibrio* spp., a higher salinity has positive effects on growth and health status of *P. monodon* (Abraham & Sasmal, 2009). A weakened innate immunity was seen in *L. vannamei* that received combined stress of a *V. alginolyticum* injection and a transfer from water with a salinity of 35‰ to water with a salinity of 15‰ (Li et al., 2010). Especially reduced total haemocyte counts, phenoloxidase activity, respiratory burst and superoxide dismutase were measured, and it was concluded that these shrimp would be killed due to decreases in their immunity and their resistance against *V. alginolyticus* (Li et al., 2010). The conclusion of these results was that shrimp farming should be maintained at a constant high salinity level to prevent exacerbated decreases in innate immunity, especially when the shrimp get infected by a pathogen, as this might lead to high mortalities (Li et al., 2010). This is supported by another study that showed higher mortalities in shrimp injected with *V. alginolyticus* and then transferred to water with a salinity of 5‰ or 15‰ compared with shrimp that were kept in water of 25‰ and 35‰ (Wang & Chen, 2005). A shift in the composition of the bacterial microbiome in the water with a lowered salinity might also contribute to a higher risk of bacterial infections in shrimp, as, for example, different *Vibrio* spp. show a different pathogenic potential for shrimp. Therefore, in the present study, the influence of a reduced salinity of 15‰ on the composition of *Vibrio* species in the water was examined and compared to the species composition at a high salinity of around 30‰. Six separate

TABLE 5 Pathogenicity factors detected in *Vibrio* species

	PIR A/B toxins	Motility/flagellins						Haemolysis/haemolysins					Pathogenicity factors of				
			Flagellins of *V. harveyi*		Flagellins of *V. vulnificus*								*V. cholerae*			*V. parahaemolyticus*	
Species	*pirAB*	Motility	*flaB*	*flaC*	*flaB*	*flaC*	*flaD*	Hae	*vhh*	*vfh*	*TDH*	*TRH*	*VPI*	*ToxR*	*ToxS*	*T6SS1*	*T6SS2*
V. alginolyticus ($n = 11$)	0/11	1.64 ± 0.92	8/11	2/11	n.t.	n.t.	n.t.	5/11	0/11	0/2	0/2	0/2	0/11	10/11	8/11	n.t.	n.t.
V. campbellii ($n = 8$)	0/8	2.75 ± 1.04	0/8	0/8	n.t.	n.t.	n.t.	0/8	0/8	0/7	0/1	0/1	0/8	0/8	0/8	n.t.	n.t
V. fluvialis ($n = 6$)	0/6	2.67 ± 0.82	1/6	6/6	n.t.	n.t.	n.t.	5/6	0/6	6/6	0/1	0/1	0/6	1/6	4/6	n.t	n.t
V. harveyi ($n = 14$)	0/14	2.29 ± 1.59	3/14	4/14	n.t.	n.t.	n.t.	3/14	7/14	0/14	0/1	0/1	2/14	0/14	0/14	n.t	n.t
V. navarrensis ($n = 6$)	0/6	4.00 ± 0.00	0/6	0/6	0/6	0/6	0/6	6/6	0/6	0/5	n.t.	n.t.	0/6	0/6	0/6	n.t	n.t
V. owensii ($n = 14$)	0/14	1.64 ± 1.15	1/14	1/14	n.t.	n.t.	n.t.	3/14	0/14	0/11	n.t.	n.t.	2/14	0/14	2/14	n.t	n.t
V. parahaemolyticus ($n = 12$)	0/12	2.33 ± 1.15	12/12	0/12	n.t.	n.t.	n.t.	11/12	0/12	0/12	0/12	0/11	0/12	12/12	9/12	1/12	12/12
V. pelagius ($n = 3$)*	0/3	1.00 ± 1.00	0/3	2/3	n.t.	n.t.	n.t.	0/3	1/3	0/2	n.t.	n.t.	0/3	1/3	0/3	n.t.	n.t
V. rotiferianus ($n = 7$)	0/7	2.00 ± 1.29	1/7	6/7	n.t.	n.t.	n.t.	1/7	0/7	0/5	n.t.	n.t.	4/7	0/7	0/7	n.t	n.t
V. tubiashii ($n = 8$)	0/8	2.88 ± 1.13	0/8	6/8	n.t.	n.t.	n.t.	6/8	0/8	0/1	n.t.	n.t.	0/8	0/8	0/8	n.t	n.t
V. vulnificus ($n = 1$)	0/1	1.00 ± 0.00	0/1	0/1	1/1	1/1	1/1	0/1	0/1	0/1	n.t.	n.t.	0/1	0/1	0/1	n.t	n.t
V. xuii ($n = 6$)	0/6	3.00 ± 1.55	1/6	4/6	n.t.	n.t.	n.t.	2/6	1/6	0/2	0/1	0/1	0/6	0/6	1/6	n.t	n.t

Note: Shown are the results for the examination of 96 *Vibrio* isolates for PIR A/ B toxins, motility, flagellins *flaB* and *flaC* of *V. harveyi*, flagellins *flaB*, *flaC* and *flaD* of *V. vulnificus*, haemolysis, haemolysins *vhh*, *vfh*, *TDH* and *TRH*, *VPI*, *ToxR* and *ToxS* of *V. cholerae*, and *T6SS1* and *T6SS2* of *V. parahaemolyticus*. The number of examined isolates per species and the pathogenicity factor are given after the forward slash in each table cell, and the number of positive tested isolates is given before the forward slash in each table cell. If at least one isolate per species and pathogenicity factor were tested positive, the numbers in the table cells are written in bold. If isolates of a certain species were not tested for a specific pathogenicity factor, "n.t." (not tested) is written in the table cell. For motility, the mean value and the standard deviation of the results from the hanging drop method are given for each species.

RAS of different scales were included in the study. The water volumes in the tanks ranged from 70 L up to 88,000 L, whereas the two commercially run systems had a higher water volume compared with the laboratory systems. Nevertheless, the total amount of bacteria in the water was in general similar in the different RAS, but slightly higher in the commercially run RAS and at higher salinities. The *Vibrio* species isolated in the present study were cultured from water samples and identified by *pyrH* or 16S rRNA sequencing. Culturing bacteria from the aquatic environment is limited as not all bacterial species grow on culture media. Nevertheless, *Vibrio* spp. grows on different media, and a clear identification on species level is possible by *pyrH* or 16S rRNA sequencing (Bauer et al., 2018). However, a species identification in *Vibrio* spp. on the basis of the brief reading obtained, for example by NGS, is often not possible (see Bauer et al., 2018). Therefore, a cultural method was performed for analysing the vibrio community. Due to the lowered salinity, the composition of the *Vibrio* species in the water in the systems changed. An altered composition of *Vibrio* species was detected also in cultured *P. monodon* that developed a disease (Sung et al., 2001). The diversity in the *Vibrio* composition decreased before the first signs of a disease were present, and especially *V. harveyi* and *V. parahaemolyticus* were detected. However, no clear relationship between the reduction in species diversity and subsequent occurrence of disease was detected (Sung et al., 2001). Changes in the bacterial composition of shrimp tank water and especially shrimp intestine were shown to be relevant for shrimp health (Xiong et al., 2015). Furthermore, it could be shown that the microbiome in a shrimp tank changes during a disease outbreak (Xiong et al., 2015). The results of the present study show that the abundance of certain *Vibrio* species was comparable in RAS operated at both salinities, whereas other species were mainly present at high or low salinity. Significant losses in shrimp, especially in larval and juvenile stages, can be experienced in aquaculture due to infections with different *Vibrio* species (Aguirre-Guzman, Ruiz, & Ascecio, 2004). *Vibrio alginolyticus*, *V. fluvialis*, *V. harveyi* and *V. parahaemolyticus* are described as pathogens for shrimp but also for humans (Austin & Zhang, 2006; Gopal et al., 2005; Igbinosa & Okoh, 2010; Li et al., 2017; Liu et al., 2004; Ramamurthy, Chowdhury, Pazhani, & Shinoda, 2014; Vandenberghe et al., 1999; Zhou et al., 2012). In the present study, *V. alginolyticus* was detected in RAS of different salinities, but more frequently in the RAS run at a salinity of 30‰. *Vibrio alginolyticus* was often used in infection experiments assecondary pathogen to investigate the effects of different water conditions on shrimp. *Vibrio alginolyticus* was able to induce clinical signs of disease and a decreased innate immunity in *L. vannamei*, but chemical and physical water parameters were decisive for disease severity (Cheng et al., 2005; Li & Chen, 2008; Li et al., 2010; Liu & Chen, 2004; Tseng & Chen, 2004; Wang & Chen, 2005). The higher amounts of *V. alginolyticus* in RAS run at a salinity of 30‰ therefore seemed not to be alarming. *Vibrio harveyi* can be regularly isolated from *P. monodon* suffering from loose shell syndrome or white gut disease, and especially isolates that induce loose shell syndrome seemed to be more virulent compared to other isolates (Jayasree, Janakiram, & Madhavi, 2006). In another study, it could be shown that sucrose-fermenting biotypes of *V. harveyi* seem to be pathogenic for *P. monodon* (Alavandi, Manoranjita, Vijayan, Kalaimani, & Santiago, 2006). In *L. vannamei*, a non-luminescent strain of *V. harveyi* was shown to induce the bacterial white tail disease (Zhou et al., 2012). Specific strains of *V. harveyi* seem to colonize the water in shrimp tanks. For instance, antibiotic-resistant strains of *V. harveyi* were found in tanks with larvae from *P. monodon*, but were neither detected in healthy eggs and nauplii nor in the intake sea water (Karunasagar et al., 1994). In the present study, *V. harveyi* was present in all examined RAS, except RAS 2 and only a few examined isolates were tested positive for the studied pathogenicity factors. In the analysis of the presence of *flaB* and *flaC* genes of *V. harveyi*, a correlation between motility and gene detection could not be seen. These results indicate that the mechanisms of bacterial motility are complex and cannot be fully explained by analysing single flagellin gene loci. Yet, isolates positive for flagellin genes might have an advantage when growing in a suitable environment, as the biogenesis of flagella requires a hierarchical system of activators and master regulators (Merino, Shaw, & Tomas, 2006). In the present study, in particular, *V. parahaemolyticus*, *V. owensii* and *V. campbellii* were found in higher numbers in water from the RAS run at a reduced salinity of 15‰ compared to the RAS run at 30‰, whereas *V. owensii* was detected only in RAS 4. *Vibrio parahaeomolyticus* is known as an opportunistic pathogen that is commonly present in sea water and brackish water. Since 2009, disease outbreaks caused by this bacterium, with mortalities up to 100%, occurred in *L. vannamei* and *P. monodon* in South-East Asia and Mexico (de la Pena et al., 2015; Han et al., 2015; Joshi et al., 2013; Lee et al., 2015; Lightner et al., 2012; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Thitamadee et al., 2016; Tran et al., 2013). The disease was first called early mortality syndrome (Phiwsaiya et al., 2017; Thitamadee et al., 2016) and is now known as acute hepatopancreatic necrosis disease (AHPND) (Han et al., 2015; Sirikharin et al., 2015). The aetiological agent is unique strains of *V. parahaemolyticus*, carrying a AHPND-associated 69-kb plasmid encoding the binary toxins *V. parahaemolyticus Photorhabdus* insect-related toxins ($PirA^{Vp}$ and $PirB^{Vp}$). These toxins are produced in the stomach of infected shrimp by the bacteria (Joshi et al., 2013; Lee et al., 2015; Sirikharin et al., 2015; Theethakaew et al., 2017; Yang et al., 2014). Recently, in non-AHPND isolates of *V. parahaemolyticus*, a 183-kb plasmid was found that shares almost identical genetic components to the 69-kb plasmid (Theethakaew et al., 2017). Additionally, it was shown that isolates of *V. parahaemolyticus* might carry a mutant pVA plasmid that does not encode the toxins $PirA^{Vp}$ and $PirB^{VP}$ and does not cause typical AHPND lesions but still can lead to mortalities of around 50% in shrimp populations. This shows that also a mutated version of the pVA plasmid in *V. parahaemolyticus*, that is not detected by a pVA-specific PCR, might be a risk to shrimp health (Phiwsaiya et al., 2017). Taken together, isolates of *V. parahaemolyticus* should be considered as a potential pathogen for shrimp, even when the common PCRs for detecting $PirA^{Vp}$ and $PirB^{Vp}$ show negative results. A large extrachromosomal plasmid, which encoded *pirAB* toxin genes, was detected also in *V. owensii* isolates that were causing AHPND in shrimp (Liu

et al., 2015, 2017, 2018; Xiao et al., 2017). The plasmid shared a high sequence similarity with that observed in *V. parahaemolyticus*. All isolates of *V. owensii* examined in the present study were tested negative for *pirAB* toxin genes, and only a few of the examined isolates were tested positive for other pathogenicity factors (*flaB*, *flaC* of *V. harveyi*, VPI and ToxS of *V. cholerae*). Nevertheless, as *V. owensii* might also carry the *pirAB* toxins and could only be detected in one of the RAS with a low water salinity of 15‰, a reduced salinity seems to be a risk factor for infections of shrimp by *V. owensii*. Homologue counterparts of the *pirAB* toxin genes were also detected in *V. campbellii* (Xiao et al., 2017), and even in archived samples of *V. campbellii*, collected 5–10 years prior to the reported AHPND outbreaks, PirA and PirB toxins were discovered (Wangman et al., 2018). In an experimental infection of *L. vannamei*, it could be demonstrated that a *V. campbellii* isolate carrying pir^{Vp} was causing AHPND in the shrimp (Dong et al., 2017). All examined *V. campbellii* isolates in the present study were tested negative for *pirAB* toxin genes and all other analysed pathogenicity factors. As also *V. campbellii* was only detected in the RAS run at 15‰, it nevertheless seems to be a potential threat to shrimp in systems with lowered salinity. None of the *Vibrio* isolates investigated in the present study were found positive for $PirA^{Vp}$ and $PirB^{Vp}$, but most of the *V. parahaemolyticus* isolates could be tested positive for a number of additional pathogenicity factors that might be relevant for human health. All isolates were tested positive for *flaB* of *V. harveyi*, *ToxR* of *V. cholerae* and *T6SS2* of *V. parahaemolyticus*. Additionally, most of the isolates also tested positive for *ToxS* of *V. cholerae* and all but one isolate showed haemolysis on blood agar. Pathogenic strains of *V. parahaemolyticus* are known to produce TDH and/or TRH (Nishibuchi & Kaper, 1995; Nishibuchi et al., 1989; Suthienkul et al., 1995), but in the present study, none of the tested isolates carried *tdh* or *trh* genes. Taken this together, all pathogenicity factors detected in *V. parahaemolyticus* and *V. owensii* may contribute to disease outbreaks in shrimp due to infections with those isolates. As these pathogenicityfactors might be also relevant for human health, isolates carrying these factors therefore may also have an impact on food safety.

In conclusion, in the present study, it could be shown that the *Vibrio* population in water of shrimp RAS differed significantly depending on water salinity. In RAS at a low salinity of 15‰, especially higher numbers of the potential pathogenic species *V. parahaemolyticus*, *V. owensii* and *V. campbellii* were detected, which might all produce PirA and PirB toxins, the aetiological cause for AHPND in shrimp. In water with a high salinity of 30‰, these species were virtually absent. In none of the isolates, *pirAB* was detected, but as multiple other pathogenicity factors were present, especially in the examined *V. parahaemolyticus* isolates, it can be concluded that a reduced salinity may lead to a shift in *Vibrio* populations towards pathogenic species and thereby increase the risk of disease outbreaks in shrimp in RAS. As some of the detected pathogenicity factors are also relevant for human health, reduced salinity might additionally contribute to reduced food safety. When considering reducing the salinity in RAS for shrimp production, not only the negative effects on shrimp innate immunity but also the shift in *Vibrio* species composition, that might even elevate the risk for disease outbreaks, should be taken into account. For a production of healthy shrimp, that can be marketed as safe and sustainable food for human consumption, keeping shrimp at high water salinities seems to be recommendable.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data used and analysed during the current study are available from the corresponding author on reasonable request. *PyrH* sequences were submitted to the BankIt database (Table S1).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Bauer J, Teitge F, Neffe L, et al. Impact of a reduced water salinity on the composition of *Vibrio* spp. in recirculating aquaculture systems for Pacific white shrimp (*Litopenaeus vannamei*) and its possible risks for shrimp health and food safety. *J Fish Dis*. 2021;44:89–105. https://doi.org/10.1111/jfd.13270