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Essential oil blend as a safe and effective disinfectant strategy for shrimp hatcheries

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Abstract

The *Vibrio* group of bacteria is considered highly pathogenic to shrimp larvae. Shrimp larvae are susceptible to *Vibrio*. It is difficult to eradicate *Vibrio* from the hatchery environment completely. Efforts were made to design an effective disinfectant with almost no side effects or residues. A formulation was developed by combining essential oils with antimicrobial properties to fight against pathogenic *Vibrios*, such as *Vibrio harveyi* and *V. parahaemolyticus*, in a hatchery environment. The anti-*Vibrio* formulation is a blend of *Eucalyptus globulus*, jasmine, and gardenia oils, designated as essential oil blend formulation (EOBF). The effective dose of EOBF was optimized on bacteria using TSA and on *Vibrio* using TCBS agar media. The optimized dose of 40 ppm EOBF was sprayed on the tank and culture area dry surfaces. The trial sites selected were the water filter tank, maturation tank, fry reservoir tank, quarantine and maturation tank, hatchery tank, spawning tank, rinsing tank, plankton tank, larvae rearing tank, packing tank, artemia tank, polychaetes (bucket and floor), squid (equipment and table), and freshwater tank of an experimental hatchery. The swab samples were collected after 3 hours, and a significant decrease in *Vibrio*, mostly green colonies, such as *Vibrio harveyi* and *V. parahaemolyticus*, was found after EOBF application. The obtained results showed that EOBF is an effective disinfectant against pathogenic *Vibrios* in shrimp hatcheries.

Keywords: Pathogenic vibrio, *vibrio parahaemolyticus*, *vibrio harveyi*, essential oil blend formulation, disinfectant. Shrimp hatchery, *penaeus vannamei*

Introduction

The *Vibrio* group of bacteria is considered the most common and pathogenic for shrimp hatcheries. Severe mortality occurs in the early stages of shrimp development, which ultimately results in heavy losses in the shrimp. *In vitro* and *in vivo* trials have shown that some *Vibrio* strains are harmful at specific temperatures in *Vannamei* and *Monodon* shrimp [1]. *Vibrio*'s natural habitat of seawater is consistently found in hatcheries in either large or small amounts and *Vibrio* acts as an opportunistic pathogen [2]. *Vibrio* may cause heavy mortality, even up to 100% in shrimp [3, 4]. It has emerged as a facultative pathogen for shrimp in combination with environmental stress [5, 6]. In this context, it is challenging to prohibit opportunistic and secondary pathogens such as *Vibrio* during the entire culture period. Chemicals or health supplements in aquatic organisms are applied to the whole population, resulting in resistant microbial strains. It can change the regular microbial composition, leading to massive outbreaks of the disease.

Due to that scenario, there is a need to have a formulation with minimal side effects and a specific target for application in a confined environment such as a hatchery. Essential oils with antimicrobial properties could fit these requirements. Essential oils (EOs) should be considered the most promising natural antimicrobials, as they do not cause microbial resistance due to their diversity of mechanisms of action. EO has a GRAS status granted by the U.S. Food and Drug Administration, indicating that they are generally recognized as safe for human consumption without limitations on intake. Moreover, they are commonly accepted by customers [7]. Oils such as eucalyptus oil, jasmine oil, and gardenia oil have anti-*Vibrio* properties. Compounds such as flavonoids and biophenols present in eucalyptus have direct microbicidal activity against bacteria [8].

Eucalyptus essential oils and their major constituents possess toxicity against a wide range of microbes, including bacteria and fungi, both soil-borne and postharvest pathogens. They have been found to reduce mycelial growth and inhibit spore production and germination [9]. Bachheit *et al.* [9] successfully demonstrated the efficacy of eucalyptus against *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus*, *Lactobacillus*, and *Staphylococcus aureus* using the agar diffusion method. The methanolic extract of leaves of *Gardenia coronaria* has shown antibacterial properties [10, 11]. Jasmine oil's antibacterial activity was successfully assessed against *E. coli* [12, 13]. Jasmine has shown efficacy as an antibacterial and antifungal agent [13, 14]. Several bacteria have been reported to have biofilm properties.

A successful effort was made to develop a blend of essential oils containing eucalyptus oil, gardenia oil, and jasmine oil to develop a safe and effective disinfectant for shrimp hatcheries.

Materials and Methods

1. Trial Station

The trial was conducted from April 2020 to August 2020 at the Marine Research Centre, PT Central Proteina Prima, Lampung, Indonesia.

2. Essential blend oil formulation (EOBF) preparation and composition

Eucalyptus globulus, jasmine and gardenia oils were obtained from vendors that comply with the strictest industry practices. Each essential oil was obtained through a steam distillation process and underwent thorough checking for the quality and chemical compositions based on the European Pharmacopeia. After the essential oils were declared to pass the quality checking, the mixture of the EOBF was created with the following sequence and percentage: *Eucalyptus globulus*, jasmine and gardenia oils were added in equal quantities to form the oil mixture and then mixed with potable water.

3. Essential blend oil (EOBF) dose optimization

The trial was conducted in two steps. The first step was used to determine the most effective and optimum dose of EOBF. The selected dose of EOBF was used in one fry rearing facility to determine the efficacy of EOBF as a disinfectant.

The first step of the trial was conducted using several doses, i.e., ten ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm. The determined EOBF dose was mixed well in water by continuous stirring for 3 to 5 minutes in a metal container. A commercially available sprayer was used to spray the EOBF

on the empty dry surfaces of hatchery tanks and reservoirs. On average, 150 to 200 ml of EOBF solution was sprayed over a 1 m² area.

The second trial step was specific, in which the determined optimum dose of EOBF was used, after which swab samples were cultured on TCBS agar to determine the amount of *Vibrio* decrease by EOBF application. The optimum dose of EOBF, 40 ppm or 40 ml per 1000 litres of water, was used in this second trial step.

4. Sample collection

The swab samples were collected before spraying with EOBF and 3 hours after the spraying to test for *Vibrio*. The swab samples were collected before EOBF application and at 30 minutes, 60 minutes, 120 minutes, and 180 minutes after EOBF application.

The swab samples were cultured on tryptose soy agar (TSA agar) from DIFCO, USA and on thiosulfate-citrate-bile salt-sucrose agar (TCBS agar) from DIFCO, USA, for the first step of the trial. The TSA was used for total bacteria counting, and the TCBS was specific for *Vibrio* bacteria counting.

5. Sample site

The EOBF was sprayed all throughout the hatchery area, and the samples were collected from similar areas. The primary locations were the water filter tank, maturation tank, fry reservoir tank, quarantine and maturation tank, hatchery tank, spawning tank, rinsing tank, plankton tank, larvae rearing tank, packing tank, artemia tank, polychaetes (bucket and floor), squid (equipment and table), and freshwater tank.

6. Disinfection process

Freshly prepared 40 ppm EOBF was used for the disinfection process. A sprayer (Sancin, China) was filled with the EOBF solution. EOBF was sprayed on empty and dry surfaces. The spray flow was mild, and the whole surface became wet. The swab samples were collected from the surface before spraying and 3 hours after spraying to evaluate the EOBF disinfectant efficacy.

Results and Discussion

1. Step 1: EOBF dose optimization

As a first step of the trial, swab samples were collected before spraying with EOBF and 3 hours after spraying. The samples were cultured on tryptose soy agar (TSA agar) and thiosulfate-citrate-bile salt-sucrose agar (TCBS agar). The results obtained from the reservoir are described in Figure 1, and the larvae rearing module is shown in Figure 2.

Table 1: Total bacterial count and total *Vibrio* count before and at different time intervals after EOBF application on the reservoir surface

AREA : RESERVOIR B											
DOSAGE (ppm)	SAMPLE	BEFORE		TBC (cfu/cm ²) IN MINUTES AFTER SPRAYING				TVC (cfu/cm ²) IN MINUTES AFTER SPRAYING			
		TSA	TCBS	30	60	120	180	30	60	120	180
10	RB.9	6.3E+00	0.0E+00	2.1E+03	0.0E+00	8.1E+01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
20	RB.10	8.8E+01	0.0E+00	3.3E+02	8.8E+01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
30	RB.11	6.3E+00	0.0E+00	6.3E+00	0.0E+00	1.1E+02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
40	RB.12	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.4E+02	1.9E+02	0.0E+00	0.0E+00	0.0E+00	0.0E+00
50	RB.13	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	4.1E+02	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Table 1 shows the presence of no *Vibrio*, which is considered harmful for the shrimp larvae, on the tank surfaces before and after application at all doses and at different time intervals.

Some unknown bacterial cultures were detected on TSA media before and after EOBF application, which may not be harmful to the larvae.

Table 2: Total bacterial count and total Vibrio count before and at different time intervals after EOBF application on the larvae rearing module surface

AREA : MODUL B											
DOSAGE (ppm)	SAMPLE	BEFORE		TBC (cfu/cm ²) IN MINUTES AFTER SPRAYING				TVC (cfu/cm ²) IN MINUTES AFTER SPRAYING			
		TSA	TCBS	30	60	120	180	30	60	120	180
10	B.31	3.8E+01	0.0E+00	0.0E+00	1.7E+01	7.5E+01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
	B.32	2.3E+01	0.0E+00	1.6E+02	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
20	B.33	6.3E+01	0.0E+00	6.9E+01	5.0E+01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
	B.34	1.1E+02	0.0E+00	2.5E+01	4.6E+01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
30	B.35	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
	B.36	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.5E+01	0.0E+00	0.0E+00	0.0E+00	0.0E+00
40	B.37	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
	B.38	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
50	B.39	6.3E+00	0.0E+00	1.3E+01	1.9E+01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
	B.40	6.3E+00	0.0E+00	1.9E+02	3.7E+02	6.3E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Table 2 shows that no Vibrio was cultured from the module's tank surfaces before and after application at all doses and at different time intervals. Some unknown bacterial cultures were detected on TSA media before and after EOBF application, which may not be harmful to the larvae. The 40 ppm dose at 180 minutes after spraying showed the best results in both, and therefore they were selected for the second trial step.

Step 2: EOBF application

The 40 ppm dose of EOBF was utilized in the second step of the trial. The swab sample was collected 180 minutes after spraying EOBF and cultured on TCBS agar. The obtained results are described below.

1.1 Overall view of Vibrio count by TCBS agar in the post-larvae production facility before and three hours after EOBF spraying

Table 3: Overall Vibrio count before and three hours after EOBF spraying in an experimental shrimp hatchery

Source	Before EOBF application			3 hrs after EOBF application		
	No. of samples	No. of +Vibrio	+Vibrio (%)	No. of samples	No. of +Vibrio	+Vibrio (%)
Maturation-Sand Filter Tank	6	0	0.0	6	0	0.0
Larva Production -Sand Filter Tank	20	0	0.0	20	0	0.0
Maturation-Reservoir & Water Chiller Tank	29	0	0.0	26	0	0.0
Larva Production -Reservoir Tank	18	0	0.0	21	0	0.0
Quarantine and Maturation Tank	26	3	11.5	26	0	0.0
Hatching Tank	30	9	30.0	30	1	3.3
Spawning Tank	24	8	33.3	9	0	0.0
Rinsing Tank	24	2	8.3	22	0	0.0
Plankton Tank	36	2	5.6	36	0	0.0
Larva rearing Tank	105	2	1.9	105	0	0.0
Packing Tank	10	0	0.0	10	0	0.0
Artemia Tank	35	0	0.0	35	0	0.0
Polychaete (bucket & floor)	6	0	0.0	6	0	0.0
Squid (equipment & table)	9	0	0.0	9	0	0.0
Fresh water Tank	16	1	6.3	20	0	0.0

The overall result obtained from the EOBF spraying is described in Table 3. A large number of samples were collected from the water reservoir, culture tank, and live food and natural food sources. The tanks, quarantine and maturation tank, hatching tank, spawning tank, rinsing tank, plankton rearing tank, larvae rearing tank, and freshwater tank showed positive samples before EOBF spraying. All the tanks were negative for Vibrio bacteria on ACBS agar after EOBS spraying.

1.2 Vibrio population measurement using TCBS agar in shrimp maturation tanks before and three hours after EOBF spraying

Figure 1 shows the presence of Vibrio bacteria in three maturation tanks, B-1, B-2, and B-3, before EOBF spraying. All the tanks showed negative samples for those collected three hours after EOBF application.

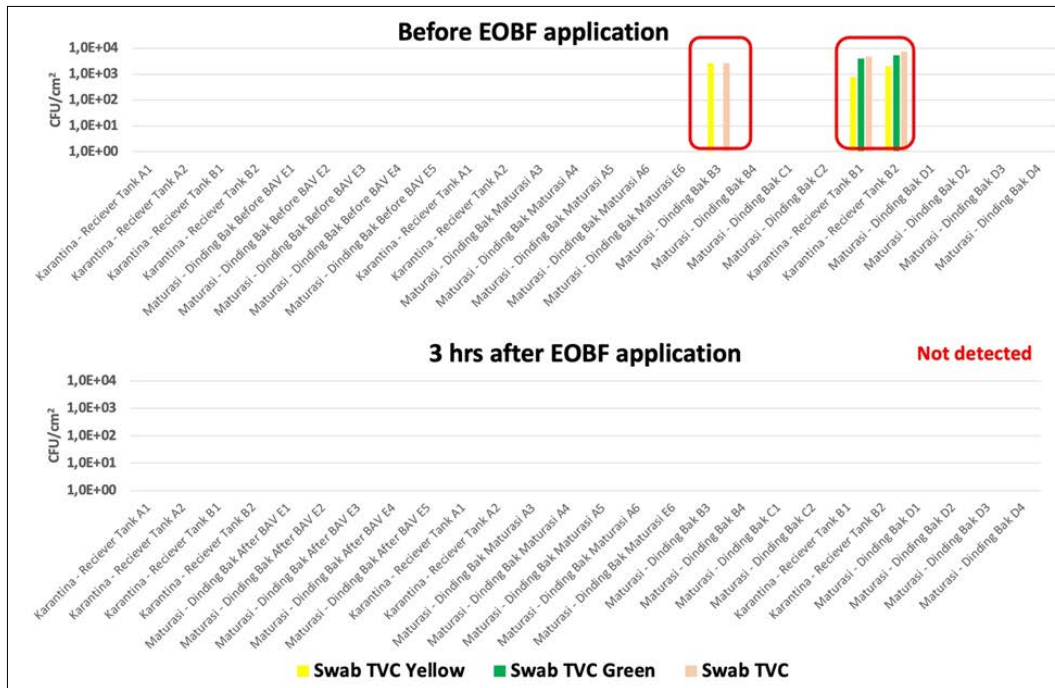


Fig 1: Vibrio count before and three hours after EOBF spraying in the shrimp maturation tanks

1.3 Vibrio population measurement using TCBS agar in the shrimp hatching tanks before and three hours after EOBF spray

Figure 2 shows the presence of Vibrio bacteria in shrimp hatching tanks 15, 21, 23, 25, 26, 28, and 30 before the EOBF spraying. All the tanks, except tank 25, demonstrated negative results in the samples collected three hours after EOBF

application. There was no green Vibrio detected in tank 25 after the EOBF spraying. Vibrio strains grown as a green colony on TCBS agar are *Vibrio harveyi*, *Vibrio campelli*, and *Vibrio parahaemolyticus*, which are considered pathogenic to shrimp [15, 16]. *Vibrio alginolyticus*, which grows as a yellow-coloured colony, is considered not pathogenic to shrimp [15, 16].

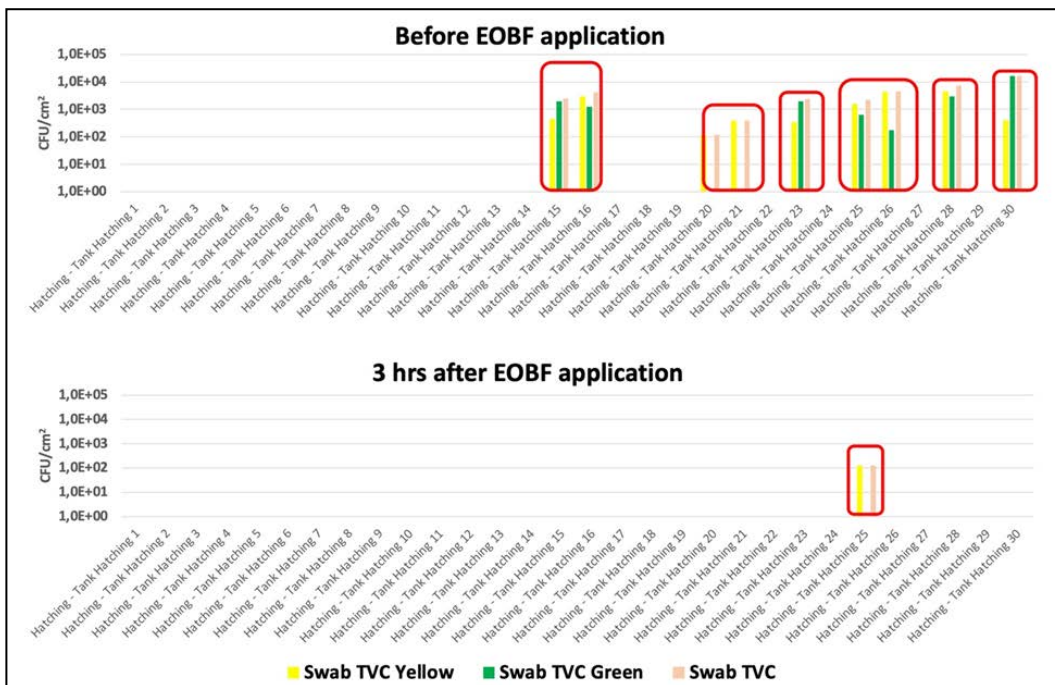


Fig 2: Vibrio count before and three hours after EOBF spraying in the shrimp hatching tanks

1.4 Vibrio population measurement using TCBS agar in the shrimp spawning tanks before and three hours after EOBF spraying

Figure 3 displays the presence of Vibrio bacteria in shrimp

spawning tanks 3, 11, 12, 15, 18, 21, 22, and 23 before EOBF spraying. All the tanks showed negative samples for those collected three hours after EOBF application.

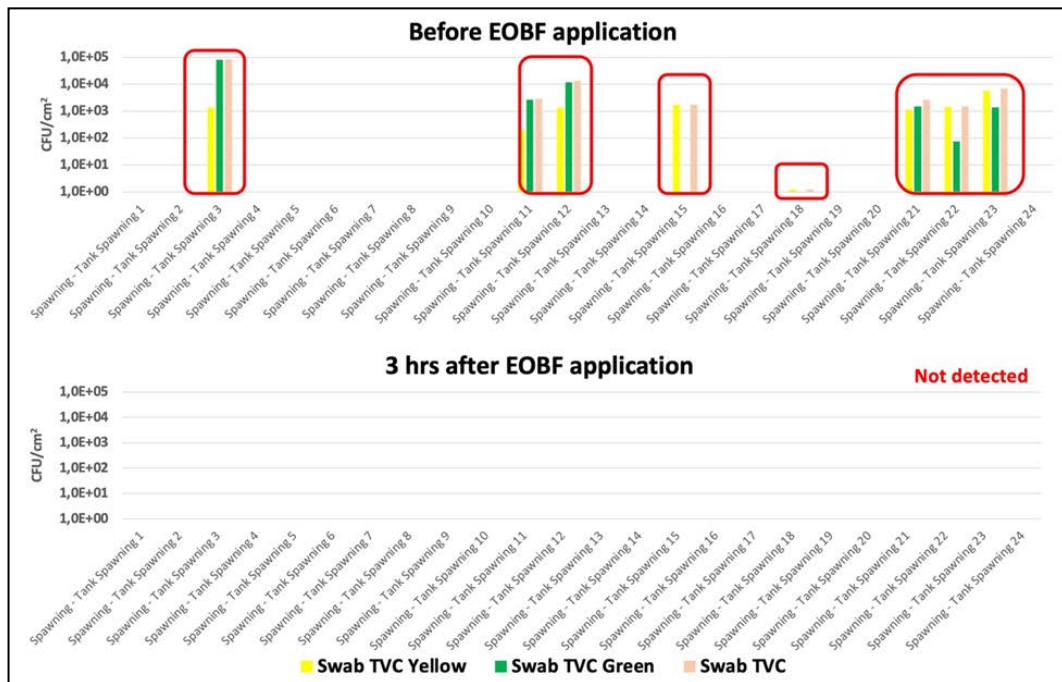


Fig 3: Vibrio count before and three hours after EOBF spraying in the shrimp spawning tanks

1.5 Vibrio population measurement using TCBS agar in the nauplii rinsing tanks before and three hours after EOBF spraying

Figure 4 shows the presence of Vibrio bacteria in nauplii

rinsing tank number 13 and 16 before EOBF spraying. All the tanks showed negative samples for those collected three hours after EOBF application.

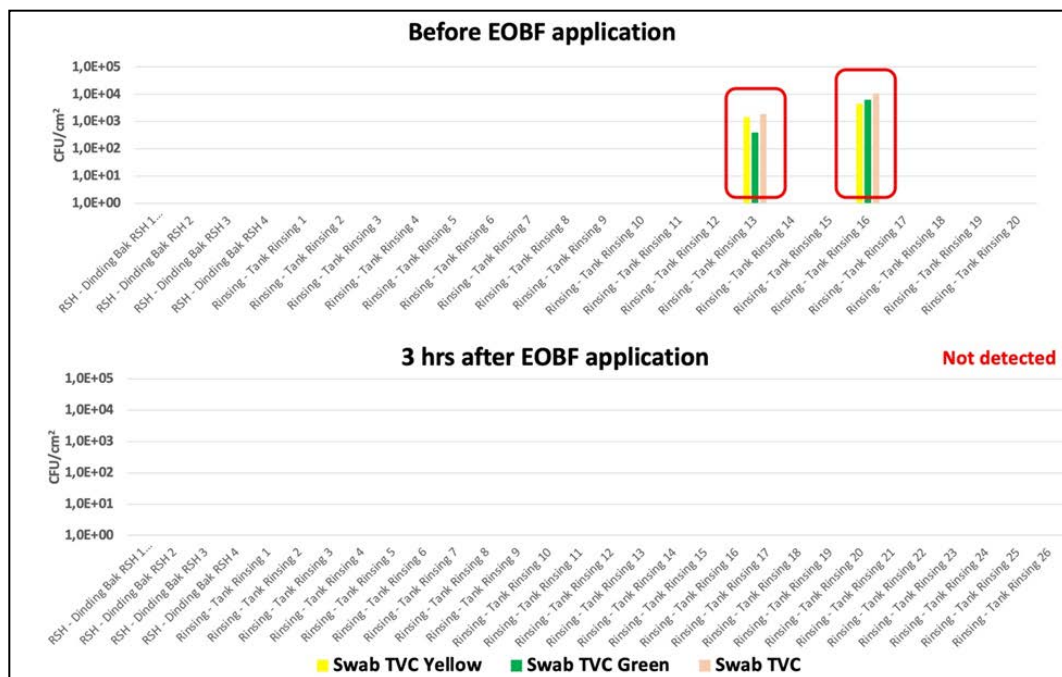


Fig 4: Vibrio count before and three hours after EOBF spraying in the nauplii rinsing tanks

1.6 Vibrio population measurement using TCBS agar in the plankton rearing tanks before and three hours after EOBF spraying

Figure 5 shows the presence of Vibrio bacteria in nauplii

rinsing tank number 7 and 16 before EOBF spraying. All the tanks showed negative samples for those collected three hours after EOBF application.

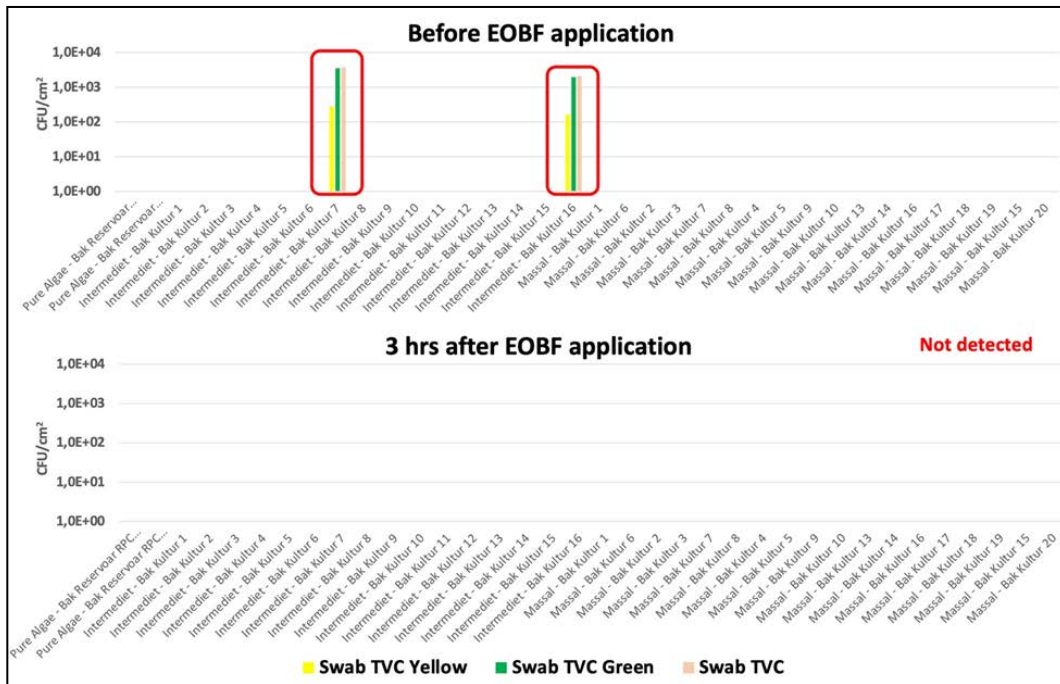


Fig 5: Vibrio count before and three hours after EOBF spraying in the plankton rearing tanks

1.7 Vibrio population measurement using TCBS agar in the larvae rearing tanks before and three hours after EOBF spraying

Figure 6 shows the presence of Vibrio bacteria in larvae

rearing tank number 19 and 21 before EOBF spraying. All the tanks showed negative samples for those collected three hours after EOBF application.

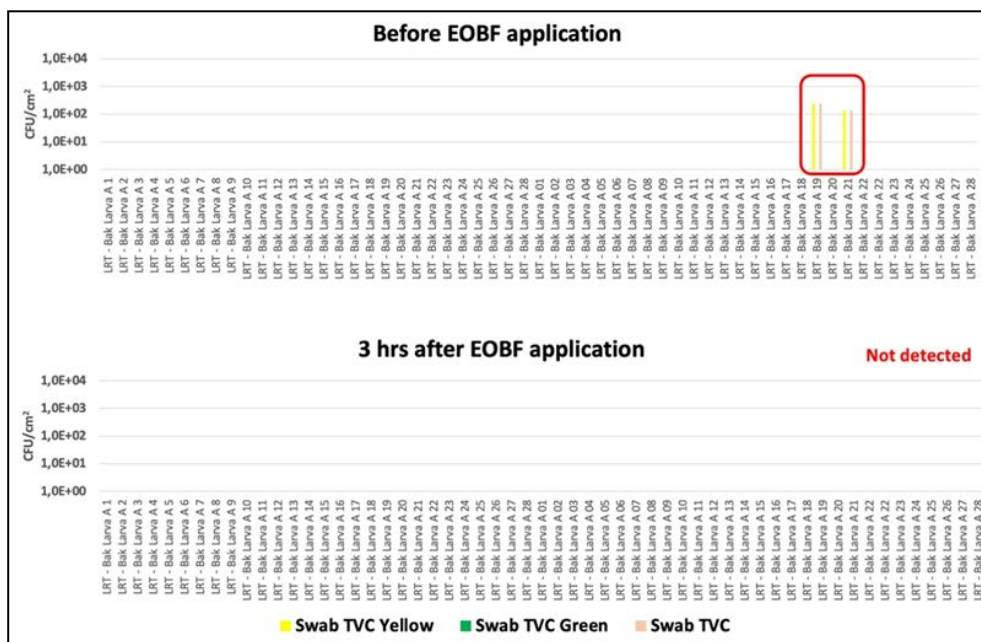


Fig 6: Vibrio count before and three hours after EOBF spraying in the larvae rearing tanks

Conclusion

The obtained results demonstrated that the essential oil blend formulation (EOBF) is a potential disinfectant against Vibrio, especially *V. harveyi* and *V. parahaemolyticus*, which are pathogenic for shrimp.

Conflicts of interest

We declare that we have no conflicts of interest.

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