

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

Rhea Natural Sciences

ECOGUARD INTRODUCTION



- *Product introduction and formulation*
- *Mode of Action*
- *Characteristics*
- *Application method and dose for shrimp farming*
- *Application method and dose for animal farming*

APPLICATION OF ECOGUARD IN SHRIMP FARMING SYSTEM



- *Introduction*
- *Recommended dose and application method*
- *Laboratory test*
- *Commercial Hatchery performance*

APPLICATION OF ECOGUARD AS POND BOTTOM DISINFECTANT



- *Introduction*
- *Recommended dose and application method*
- *Laboratory test*
- *Commercial farm performance*

CONCLUSION



- *Efficacy of Ecoguard*

- This product functions as disinfectant to deactivate and degenerate pathogens present in the aerosol, free form and attached at the surface of the floors, warehouse and storage.
- It can also serve as disinfectant when used in washing the necessary equipment.
- Unlike other disinfectant products that are usually toxic, Ecoguard is safe both for humans and animals.



The selection of blend oils was carried out based on their anti-viral - and immunomodulating properties, as well as their compatibilities.

The targeted active ingredients were as followed:

- Eucalyptus : Cineol
- Gardenia : Linalool
- Jasmine: Benzyl acetate

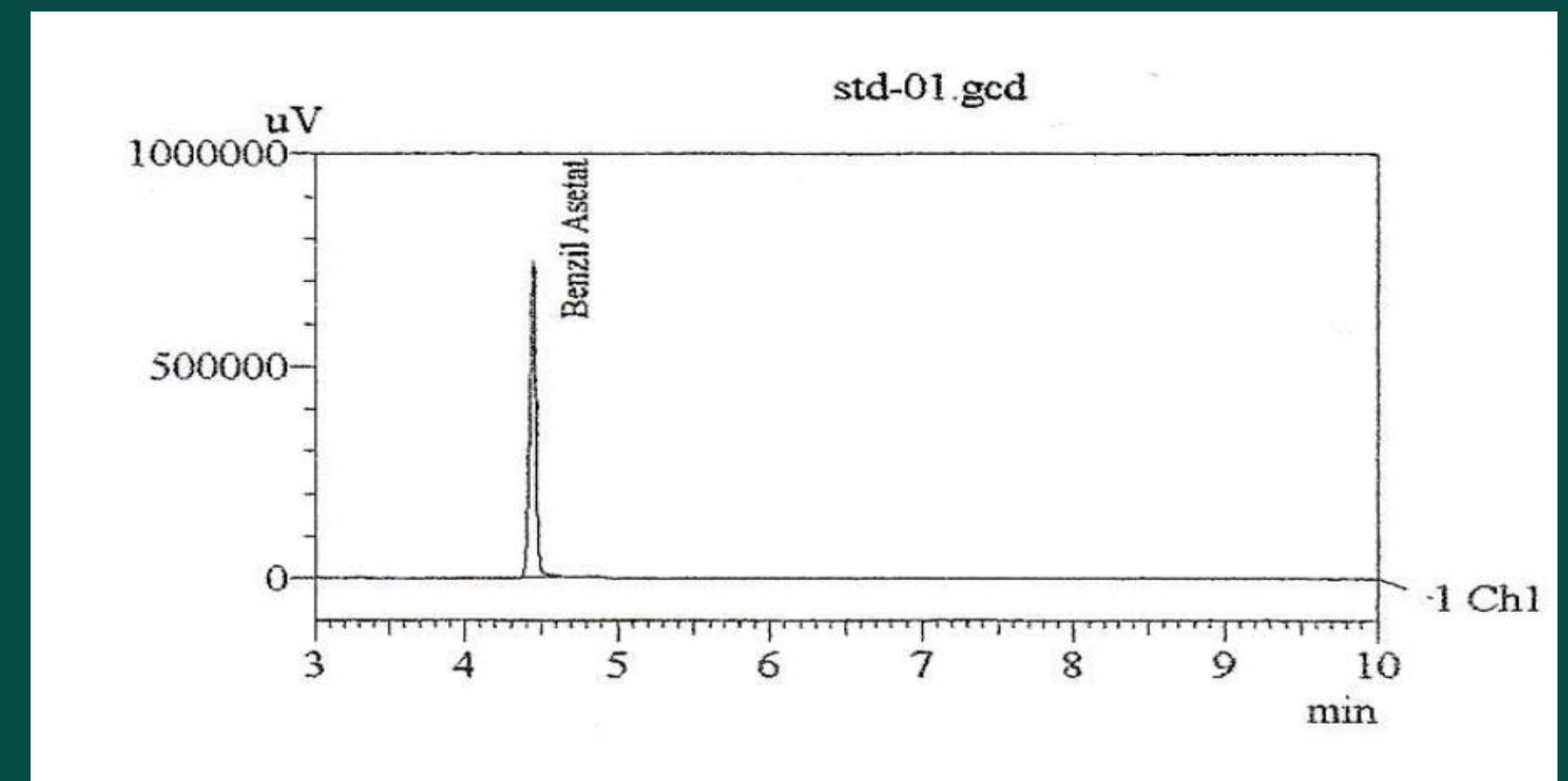
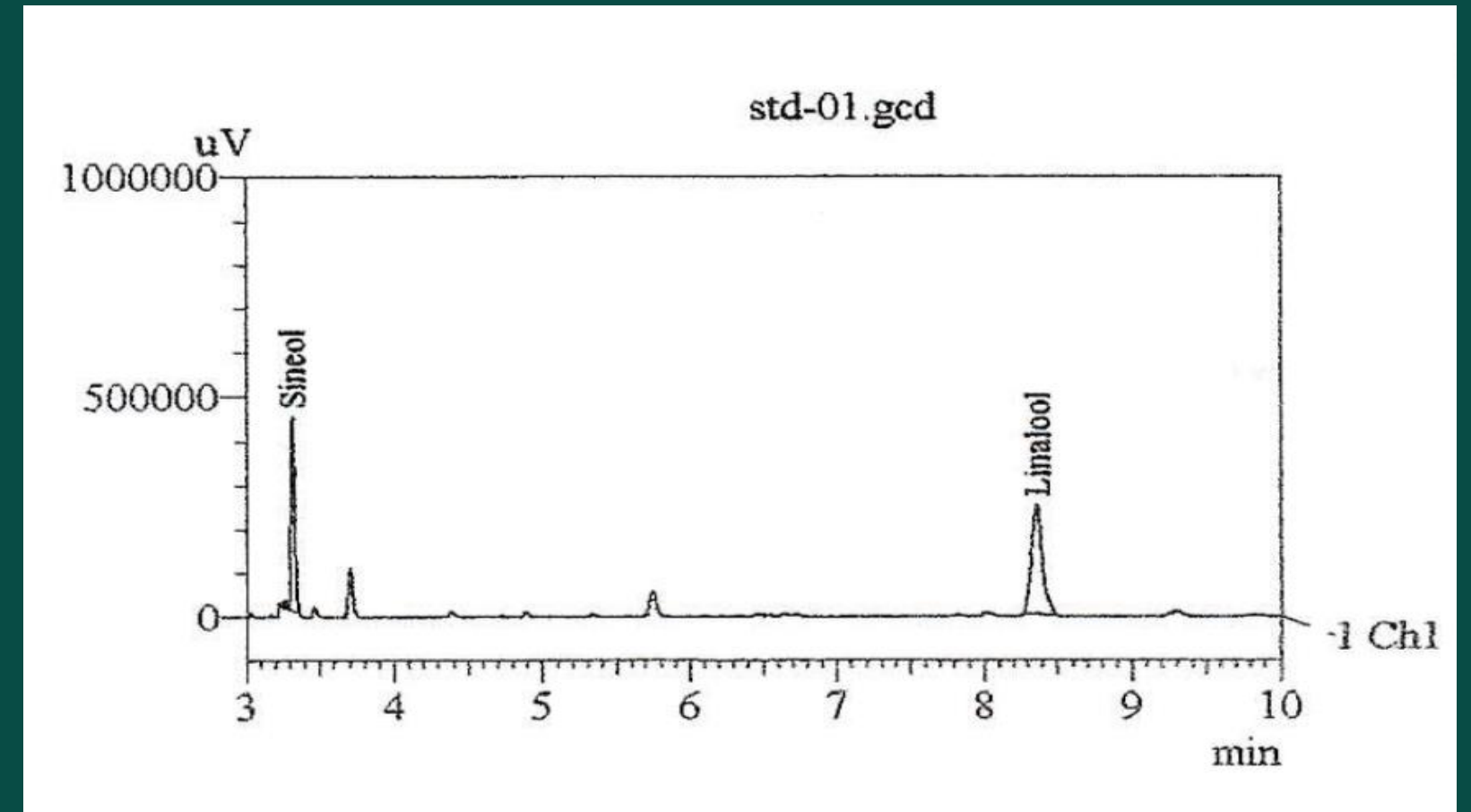


Figure: Chromatogram of complete Ecoguard's compound peak

EOs, including eucalyptus oil, jasmine oil, and gardenia oil, were selected for testing against *Vibrio*.

- The presence of flavonoids and biophenols in Eucalyptus results in microbicidal activity against bacteria.
- Leaf extracts of gardenia possess antibacterial properties.
- Jasmine oil is effective against bacteria .

A successful effort was made to develop a blend of essential oils comprising eucalyptus-, gardenia-, and jasmine oil to establish a safe and effective disinfectant to minimize luminescent pathogenic *Vibrio*

CHARACTERISTICS OF ECOGUARD

Yellow-transparent liquid

1

2

pH 3.89 – 3.92

5

Glass bottle 100 mL, 250 mL,
500 mL, and 1L



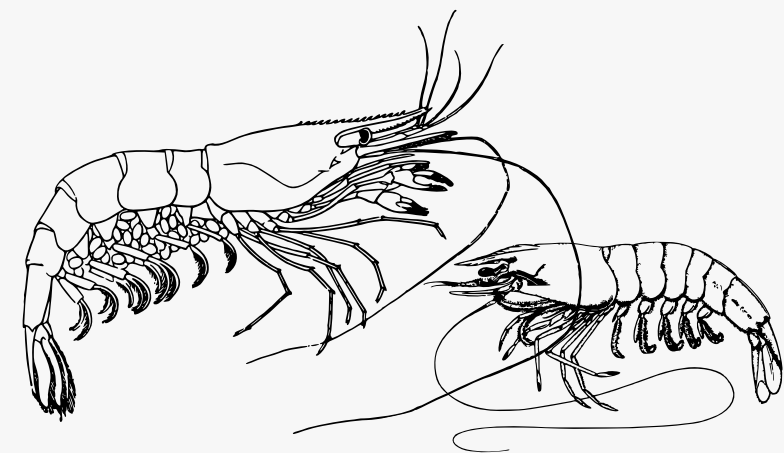
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Non-hazardous, user-friendly

3

Boiling point 197° C

ECOGUARD APPLICATION IN SHRIMP HATCHERY SYSTEMS



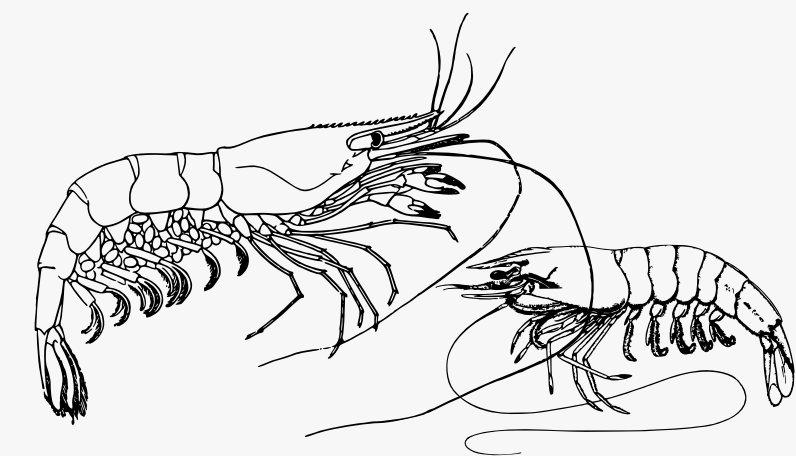
Application Of Ecoguard as Disinfectant in Shrimp Hatchery

- Luminescent or glowing bacteria is the common terminology for harmful Vibrio bacteria in shrimp hatcheries.
 - The most pathogenic Vibrio group is *Vibrio harveyi*, followed by *V. campbellii*, *V. splendidus* and *Vibrio parahaemolyticus* etc.
 - Heavy mortality, even up to 100% in shrimp, can be caused by pathogenic Vibrio in combination with environmental stress.
 - After the complete ban and prohibition of antibiotics in hatcheries, as pathogenic bacteria quickly become resistant to commercial probiotics, there is a need to obtain a remedy with minimal side effects that is equally effective against luminescent Vibrio .
- EOs with antimicrobial properties should be considered a compelling candidates against luminescent bacteria.
 - EOs are recognized as safe for human and animal consumption, as they have been granted GRAS status by the U.S. FDA.

RECOMMENDED DOSE of APPLICATION



Application Method		Dose	Frequency
SPRAYING		80 ppm	2 – 3 times a week
Application to the hatchery tanks	Preventive	Mysis tank : 3 ppm PL's Tank : 5 ppm	Every 3 rd day
	Treatment	Mysis tank : 3 ppm PL's Tank : 5 ppm	everyday



ECOGUARD SPRAYING PROTOCOL (TANKS)

Culture tank, water tanks and live-feed tank were cleaned and washed as per hatchery SOP

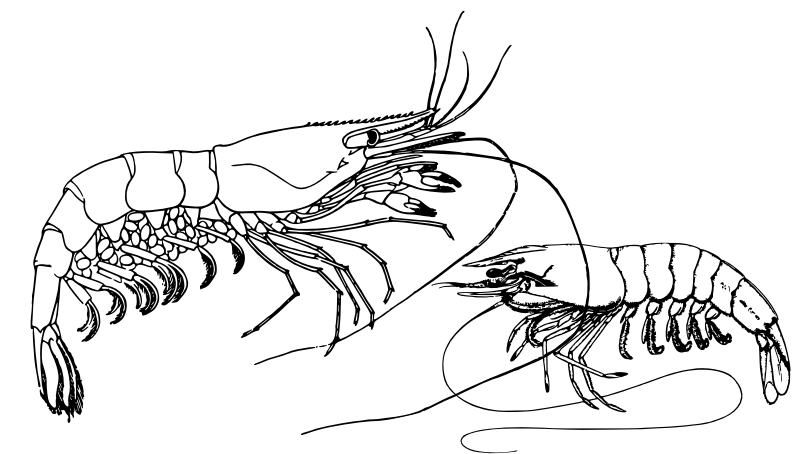
Clean and clear freshwater was prepared

80 ppm Ecoguard (0.8 mL for 10 liter of clean and clear water) was prepared and filled in a clean sprayer

Sprayed on the walls & floor of the tank until its completely wet

Left for at least 3 hours

The tank is ready to fill up the water



ECOGUARD SPRAYING PROTOCOL (Whole Hatchery Facility)

Clean and clear freshwater was prepared



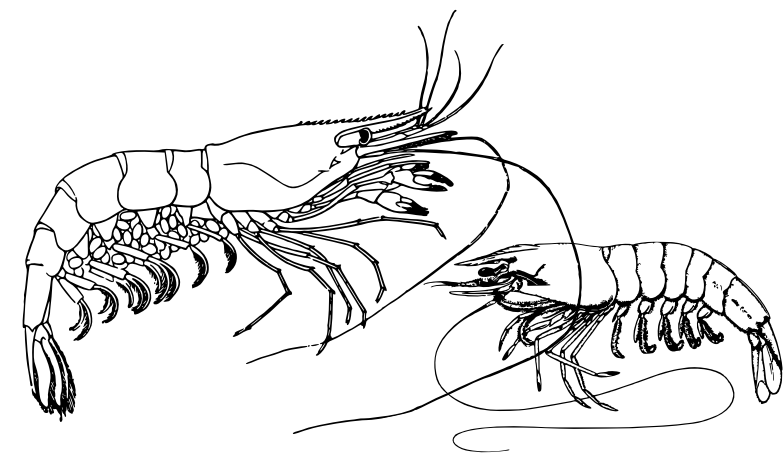
80 ppm Ecoguard (0.8 mL for 10 liter of clean and clear water) was prepared and filled in a clean sprayer



Sprayed on the hatchery gates, roof, walls, office, lab, and the whole area of hatchery until it completely wet.



This steps can be repeated for 2 times



SUMMARY OF LAB AND FIELD TRIALS

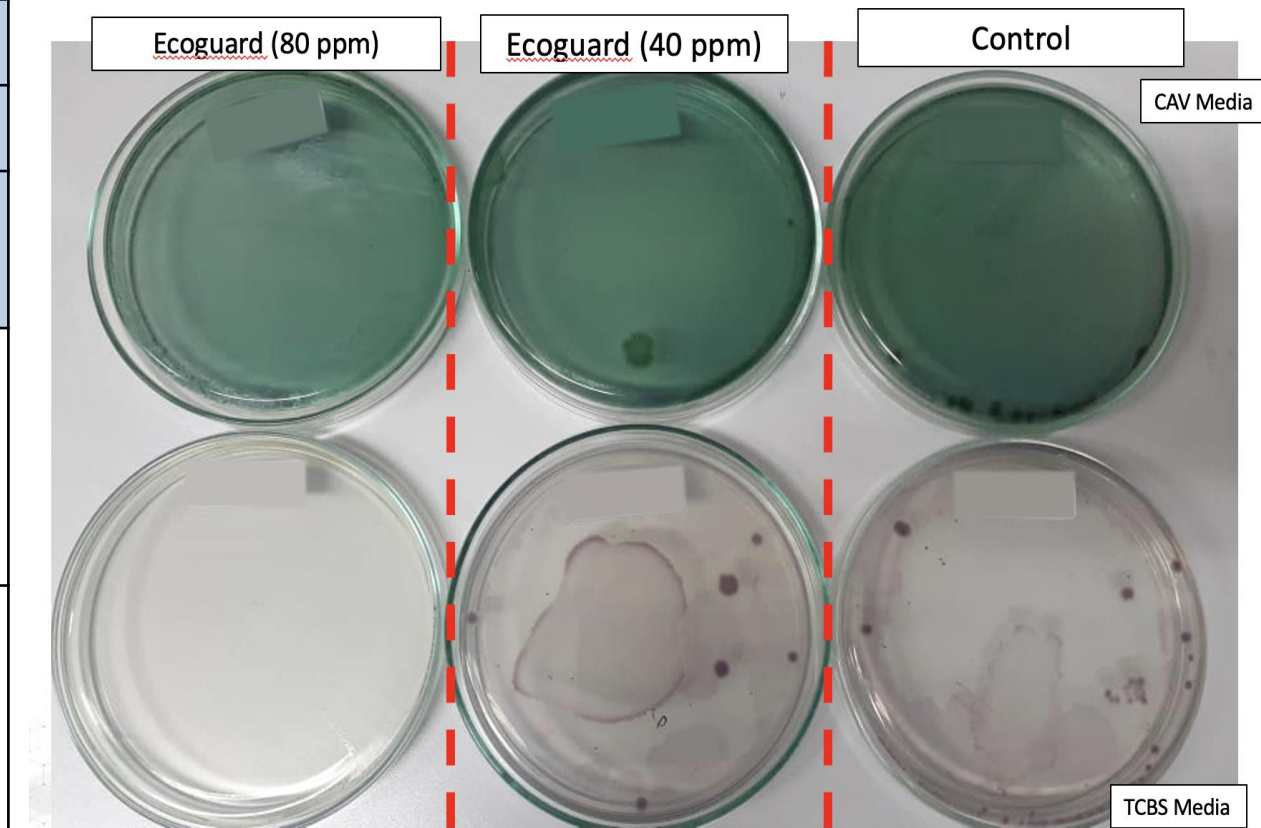
Summary of trials and initiatives

Beneficial Properties	Test Methodology	Results
Ecoguard as anti-Vibrio in water	In vitro--Indonesia, India, Saudi, Vietnam and China	Effective in reducing pathogenic Vibrio
Ecoguard as anti-Vibrio at surface & as aerosol	In Vivo-Indonesia, India, Saudi, Vietnam and China	Effective in reducing pathogenic Vibrio from tank surface and walls of the hatchery
Ecoguard as anti-AHPND	In vivo--Vietnam	Effective, 100 % effective in preventing AHPND/EMS transmission in shrimp
Ecoguard in enhancing productivity	Field observation-Indonesia, India, Saudi	Ecoguard application improved the post larvae production in shrimp hatchery by reducing the pathogen load
Ecoguard as Pond Bottom Disinfectant	Field observation-Indonesia, India, Saudi	Ecoguard application helped in reducing the pathogen load in the culture ponds

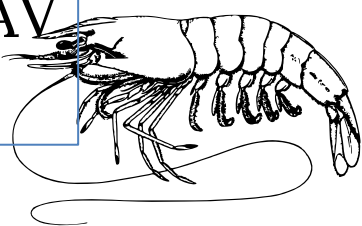
Vibrio Counting Before and After Ecoguard Application

Number of Vibrio colony before and after Ecoguard application

Group	R	Growth of <i>Vibrio parahaemolyticus</i> after Ecoguard Spray			
		TCBS media		CAV media	
		Growth	Number of Colony	Growth	Number of Colony
Control	1	+	13	+	21
	2	+		+	
	3	+		+	
40 ppm of Ecoguard	1	+	1	+	5
	2	+		+	
	3	+		+	
80 ppm of Ecoguard	1	-	0	-	0
	2	-		-	
	3	-		-	



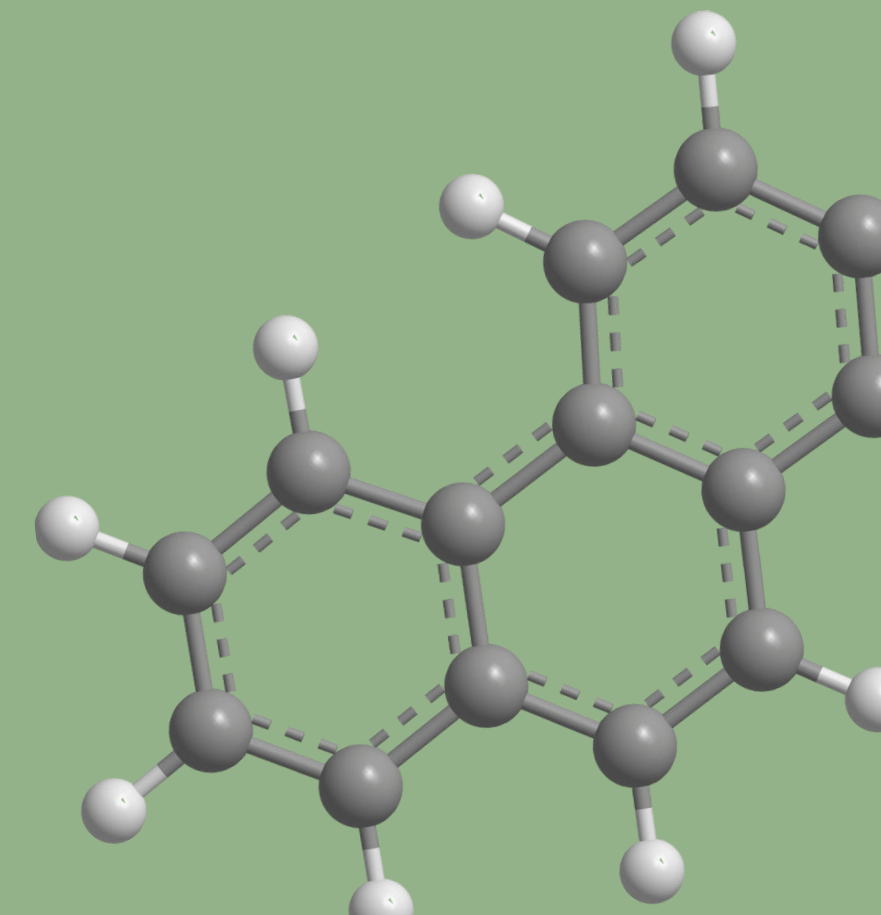
- An in vitro level trial was conducted to determine the best optimum dose of Ecoguard against *Vibrio parahaemolyticus* bacteria in a hatchery environment.
- An empty Post larvae (PL) tank was selected for the study. Ecoguard was sprayed using two fixed doses, i.e., 40 ppm and 80 ppm in the marked 16 mm² areas. The swab samples were collected before spray and after 3 hours of spray from the same area.
- The dose of 80 ppm showed the best results with zero *Vibrio parahaemolyticus* growth on both TCBS and CAV media.



Commercial Hatchery Trial Performance

TRIAL METHODS :

1. The tested tanks were cleaned and washed.
2. Before spraying, samples were taken from the tanks by swab to determine the initial number of Vibrio.
3. Ecoguard (80 ppm) was prepared and sprayed on the tank's surfaces (wall and bottom).
4. After 3 hours of spray, samples were collected to determine the efficacy of Ecoguard.



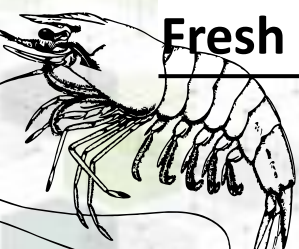
OBSERVATION 1 - CPP Indonesia

Green Vibrio reduction

Vibrio screening in Swab samples before and after spraying with 80 ppm EcoGuard

Source	Before			After		
	No. of samples	No. of +Vibrio	+Vibrio (%)	No. of samples	No. of +Vibrio	+Vibrio (%)
MNPD-Sand Filter Tank	6	0	0.0	6	0	0.0
FPD-Sand Filter Tank	20	0	0.0	20	0	0.0
MNPD-Reservoir & Water Chiller Tank	29	0	0.0	26	0	0.0
FPD-Reservoir Tank	18	0	0.0	21	0	0.0
Quarantine & 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
LRT 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

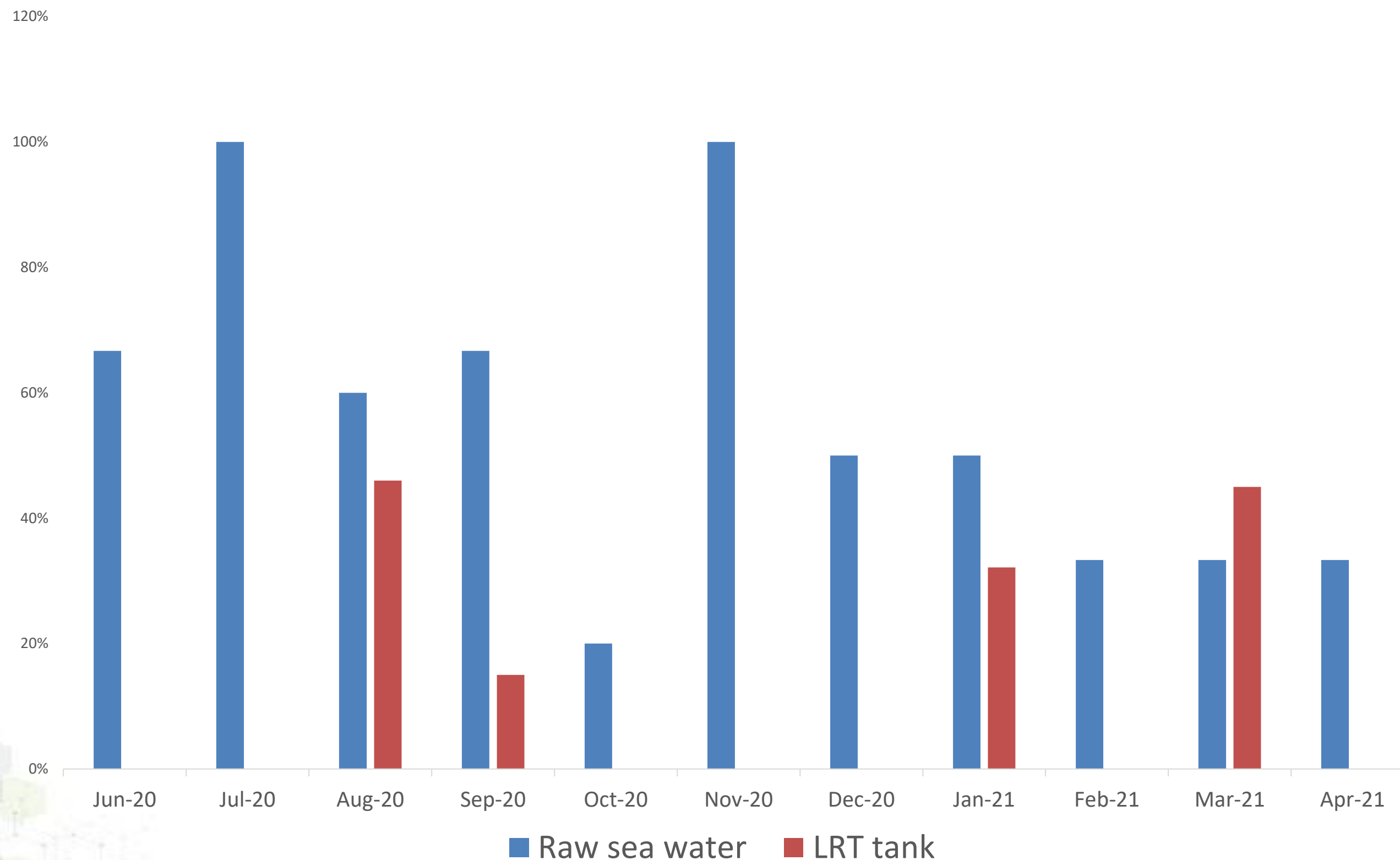
- Ecoguard dose, 80 ppm was sprayed all over the facilities of the shrimp hatchery as a disinfectant before filling up the water.
- The swab samples were collected and tested before and after the spray in the same area.
- The obtained results showed that there was up to 100 % reduction in the Vibrio colonies after Ecoguard spray



OBSERVATION 2 - CPP Indonesia

Glowing Vibrio reduction

Lumbac Incidence

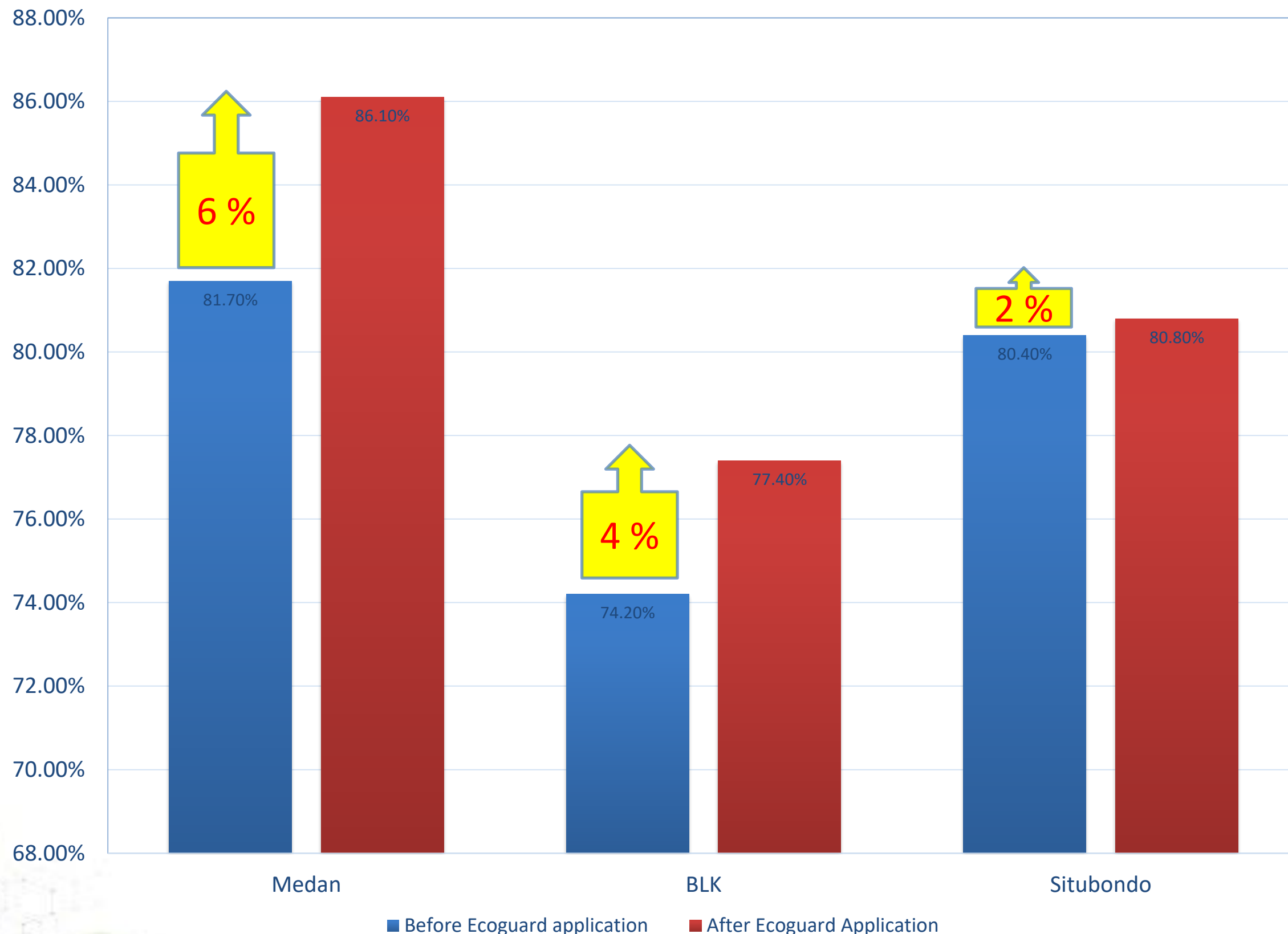


- This study was performed between June 2020-April 2021.
- Luminescent Glowing Vibrio screening was performed before and after EG spray in the Raw sea water of reservoir and Larvae rearing tank.
- The luminescent bacteria was detected in all the months in the raw water whereas the incidence was significantly lower in the larvae tanks which were disinfected by Ecoguard

OBSERVATION 3 - CPP Indonesia

Productivity improvement

Summary of PL SR (%) Jan 2020 - March 2021



- Experiment was conducted in the commercial hatcheries of PT. CP PRIMA since January 2020 to April 2021,
- Survival rate of Post Larvae (PL) as one of the indicators.
- The data shown are of three main hatcheries in Indonesia.
- There is significant increase in the survival rate up to 7% in the hatcheries after Ecoguard spray.

OBSERVATION 4 – Ecoguard Overseas performance

Productivity improvement and pathogen reduction

Parameters	Saudi	India	China	Vietnam
Target species	Post larvae of Vannamei	Post larvae of Vannamei	Post larvae of Vannamei & Fish Fry	Post larvae of Vannamei & Pig farm
Target pathogen	Pathogenic Vibrio	Pathogenic Vibrio	Pathogenic Vibrio & Streptococcus	Pathogenic Vibrio, E. coli & Salmonella
Key Performance	SR 11 % higher using EG.	SR 9 % higher using EG	Pathogenic Bacteria reduced	No Salmonella and E. coli in EG spray
Overall comments	No Green Vibrio after spray in Pond bottom & Hatchery tanks. Going to use in commercial hatchery and shrimp ponds	No Green Vibrio after spray Hatchery tanks. Started Commercial level trial	No Green Vibrio after spray Hatchery tanks. Restarted the trials	Showing good results against Salmonella & E. coli in pig & Poultry farms. Trials in shrimp hatchery will start after locked down
Ecoguard Uses	Spray at surface and apply in water	Spray at surface and apply in water	Spray at surface and apply in water	Spray at surface

CONCLUSIONS

- **Ecoguard can protect shrimps from various pathogenic Vibrio including *V. harveyi* and *V. parahaemolyticus* and other bacteria.**
- **It can improve and enhance the productivity of Post larvae**
- **It is an effective and user-friendly disinfectant**



TRIALS DOCUMENTARY INDONESIA



TRIALS DOCUMENTARY

INDIA



< 54



Surendran Mr Vai...

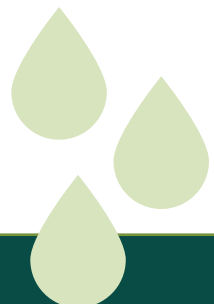
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Sat, 4 Sep

Greetings Dr. Rajeev!
Survival was 60% in the 4 LR tanks sprayed with 'essential oil'; whereas other tanks it was 54%.

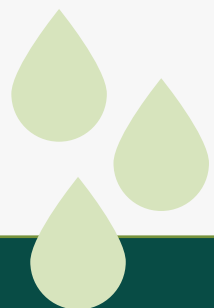
ECOGUARD APPLICATION AS POND BOTTOM DISINFECTANT



Application Method	Dose	Frequency
SPRAYING	80 ppm	Spray 1 -2 times before filling up the water

ECOGUARD APPLICATION IN ANIMAL FACILITIES

RECOMMENDED DOSE of APPLICATION



Application Method	Dose	Frequency
SPRAYING	80 ppm	2 – 3 times a week

ECOGUARD SPRAYING PROTOCOL

Rearing pens and cages, water tanks and warehouse , feeding equipment, trollies were cleaned and washed as per farm SOP



Clean and clear freshwater was prepared



80 ppm Ecoguard (0.8 mL for 10 liter of clean and clear water) was prepared and filled in a clean sprayer



Sprayed on the surface of cages and pens, walls & floor of the tank until its completely wet



Spray 2 to 3 times per week

In vitro trials of Ecoguard antiviral activity against African swine fever virus strain, Vietnam

No	Group	Dilution	HAD ^B	
			10 ⁵ HAD ₅₀ /ml	10 ⁴ HAD ₅₀ /ml
1	<u>Ecoguard</u>	1/2	0/3	0/3
2		1/4	0/3	0/3
3		1/8	0/3	0/3
4		1/16	0/3	0/3
5		1/32	0/3	0/3
6		1/64	0/3	0/3
7		1/128	0/3	0/3
8		1/256	0/3	0/3
9		1/512	0/3	0/3
10		1/1024	0/3	0/3
11		1/2048	2/3	0/3
12		1/4096	3/3(+)	2/3
13		1/8192	3/3 (+)	2/3(+)
14	Group 2: Positive control		3/3 (++++)	3/3 (++++)
15	Group 3: Negative control		0/3	0/3

- The in vitro level trial was conducted at VNUA, Vietnam to determine the efficacy of Ecoguard against ASFV.
- The various dilutions of Ecoguard were tested against log 4 and log 5 of the African Swine Fever Virus (ASFV).
- The obtained results showed that Ecoguard was effective up to dilution 10 against log 5 and dilution 11 against log 4 of ASFV.

Note: ^α All dilutions were tested in duplicate; Ecoguard (EG) was prepared as serial 2-fold dilutions; ^β, Hemadsorption (HAD) results: +: few rosette formation, ++: hundreds of rosette formation, +++: thousands of rosette formation; ++++: uncountable.

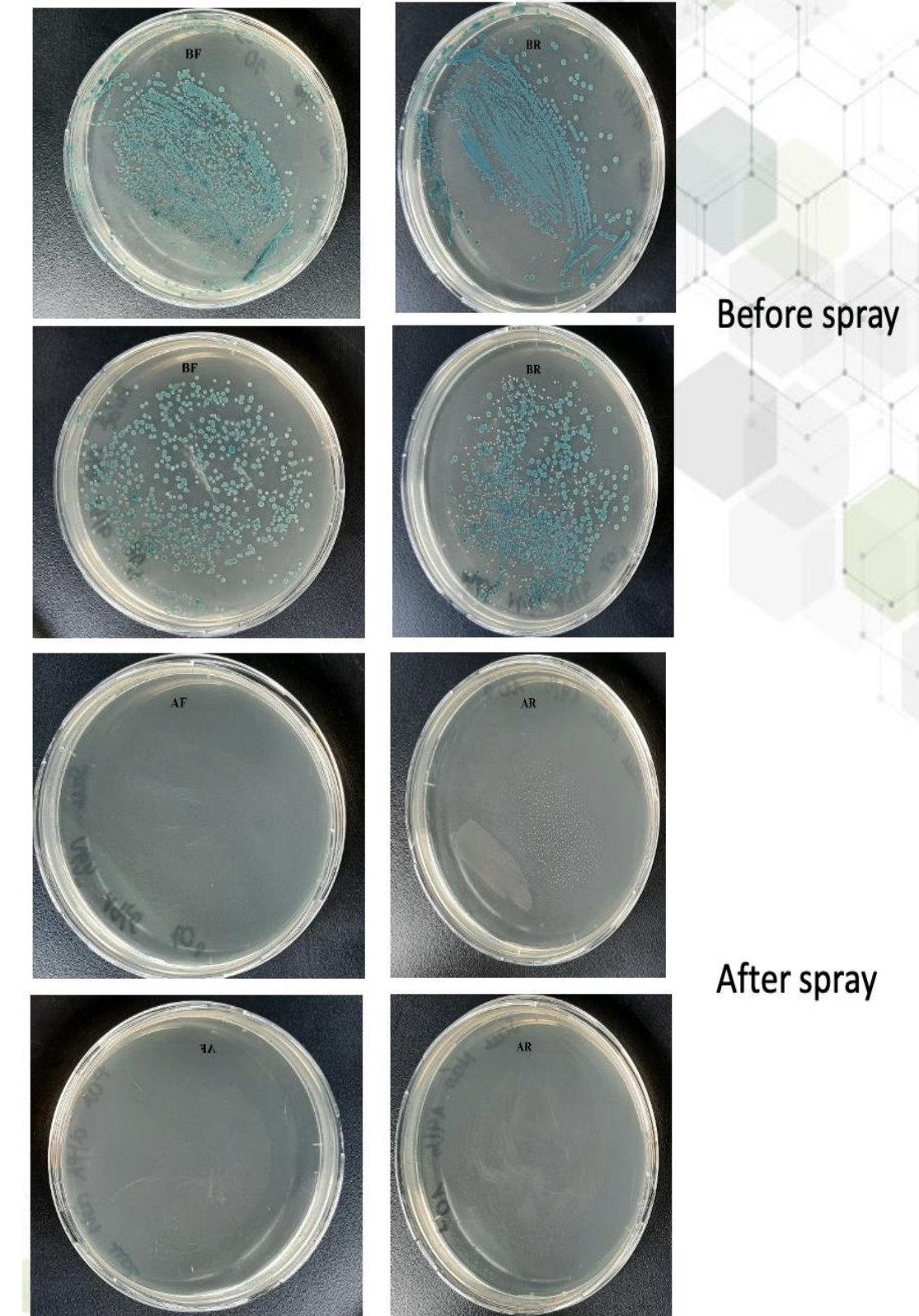
In vitro trials of Ecoguard antiviral activity against African swine fever virus strain, Vietnam

EG dilution 10 – 36h (Rosetta -)	EG dilution 11 – 96h (Rosetta +)
EG dilution 12 – 96h (Rosetta ++)	EG dilution 13 – 96h (Rosetta +++)
Negative control – 96h	Positive control – 96h

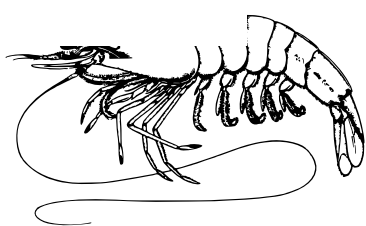
- The in vitro level trial was conducted at VNUA, Vietnam, to determine the efficacy of Ecoguard against ASFV.
- The various dilutions of Ecoguard were tested against log 5 of the African Swine Fever Virus (ASFV).
- The rate of infection was observed by the quantitative presence of hemadsorption (HAD) or Rosetta. The higher the severity the higher the number of HAD. The positive control had the highest number of HAD, whereas no HAD formation was in the negative control, and EG applied cells up to dilution 10. EG solution at dilution 11 had few HAD, which increased in dilution 12 and 13.
- The obtained results showed that Ecoguard is effective against ASFV.

Salmonella checking from swab sampling (June 14,2021)

List of samples	Swab Samples at Pig farm	Salmonella (cfu/ml) (Before EG spray)	Salmonella (cfu/ml) (After EG spray)
1.	Floor	4.6×10^7	0
2.	Wall 1	7.2×10^5	0
3.	Wall 2	2.3×10^7	0
4.	Ceiling	6.0×10^7	0



- Ecoguard spray was tested in pig farms along with Naturalguard
- The amount of Salmonella bacteria was quantified before and after treatment with EG spray
- The trial results showed that EG was able to reduce the Salmonella load from high amounts (log 7) down to zero (uncountable).



TRIALS DOCUMENTARY





MEEGID XV 15th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases ONLINE Live and On-demand 2-5 November 2021

MEEGID XV 15th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Disease - MEEGID XV (Online and On-Demand) Submission ID 29 Title Natural oil blend formulation (NOBF) protects Penaeus vannamei Boone, 1931 from white spot syndrome virus (WSSV) and enhances the productivity in the culture ponds Abstract White spot syndrome virus (WSSV) is lethal in penaeid shrimp. Successful efforts were made to develop a natural oil blend formulation (NOBF) with anti-WSSV properties using Eucalyptus globulus, Pinus sylvestris, and Lavandula latifolia in an equal proportion of water at a ratio of 1:1. A bioassay challenge trial was conducted using 1 g of 144 specific pathogen-free Penaeus vannamei Boone, 1931 samples in 4 aquarium replicates for each group. A NOBF dose of 0.2 ppm was applied throughout the trial period by mixing in aquarium water daily, starting seven days before challenge. The efficacy of NOBF against WSSV was measured using a modified per os method of challenge demonstrated in the current work. The cumulative mortality in the positive control group reached 89.6 % ten days post challenge. NOBF was also applied in six commercial shrimp ponds in a WSSV-prone area in East Java, Indonesia. Each pond was of equal size, 1000 m² and 1.2 m depth. The pathogenic Vibrio count of pond water was acceptable (<1000 cfu.mL⁻¹). The NOBF-applied ponds had better productivity (control 14.239 ton.ha⁻¹ and NOBF ponds 15.421 ton.ha⁻¹). The trial outcomes show that NOBF is safe and user-friendly, with properties that reduce WSSV load.

MEEGID XV 15th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Disease - MEEGID XV (Online and On-Demand) Submission ID 28 Title Identification of African Swine Fever Virus DNA polymerase X potential inhibitors in Natural oil blend formulation by Structure-Based Virtual Screening Approach. Abstract In this study, virtual screening approach was used to investigate Natural oil blend formulation (NOBF) individual compounds against African Swine Fever Virus (ASFV) DNA polymerase X (DNApolX). Using a combination of bioinformatics and computational tools, we predicted the interaction of dGTP binding pocket in the active site of ASFV DNApolX with 9 known constituted small molecules of blended essential oil formulation as well as natural ligand dGTP and known ASFV replication inhibitors roseterol and roseterolol as the reference compounds. We found that 91 docked small molecules presented with common amino acid residues in the dGTP-binding pocket of ASFV DNApolX with high docking score and HF score value. hotspot residues of the enzyme (Fig. 4). Fig. Molecular interactions of reference molecules dGTP, oxyclohexanol and roseterol with ASFV DNApolX hotspot residues. Superimpositions of hotspot amino acid residues on the binding site structure-based model of the ASFV DNApolX and surface exposed amino acid residues are shown. Fig. 2D Interactions of dGTP with ASFV DNApolX. dGTP interacts with the hotspot residues of the PolX and forming van der Waals bounds with Val37 and Phe116 surface exposed hotspot amino acid residues. Unfavorable donor-donor and alkyl interactions formed with Ser99 and Val120.

MEEGID XV 15th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Disease - MEEGID XV (Online and On-Demand) Submission ID 30 Title Development of essential oil blend formulation as a disinfectant against African Swine fever virus (ASFV) agent in PAM cells of Swine Abstract African swine fever virus has a significant impact on swine production and the economics of the swine-producing countries. The role of strict biosecurity measures becomes critical when there are no known remedies yet. Disinfection is considered a significant part of biosecurity measures and plays a vital role in reducing the risk of contaminating the environment. Successful efforts have been made to optimize a formulation Essential Oil Blend (EOB) product to determine its efficacy against the African swine fever virus (ASFV) in vitro conditions. The Essential Oil Blend (EOB) comprises a blend of, i.e., Eucalyptus oil, Gardenia oil, and Jasmine oil. The in vitro trial results demonstrated that EOB up to dilution ten could degenerate the lethal dose log 5 of ASFV. The work was shown by observing hemadsorption (Rosetta formation) and conducting a real-time PCR test. There was no Rosetta formation up to dilution 11 of EOB. The Ct value of the EOB group at 96 hours post-infection (hpi) was the same as the initial value or lower (25) than it, whereas the Ct value of positive control increased several folds (17.84). It is a potential water supplement to work against ASFV and enhance pig immunity to fight against common pathogens.

INTRODUCTION: AFRICAN SWINE FEVER VIRUS (ASFV) is caused by African Swine Fever Virus (ASFV). THE VIRAL GENOME: It has the ASFV genome. RESULTS & DISCUSSION: Molecular interactions of reference molecules dGTP and roseterol with ASFV DNApolX hotspot residues. CONCLUSIONS: Successful efforts have been made to optimize a formulation Essential Oil Blend (EOB) product to determine its efficacy against the African swine fever virus (ASFV) in vitro conditions.

06. Virus Evolution. POND GUARD. Chair: Martine Peeters. Speakers: Rajeev Kumar Jha, Takuya Kawahata, Mariana Kikuti, Maylis Layan, Marie Claire Morley, Alessandra Mozzi.

Posters. [P01] Comparative analysis of the protein expression in mosquitoes Aedes aegypti infected with... [P02] Identification of African Swine Fever Virus DNA polymerase X potential inhibitors in Natural oil blend formulation... [P03] Development of essential oil blend formulation as a disinfectant against African Swine fever virus (ASFV) agent in PAM cells of Swine.

[P03] Development of Essential Oil Blend formulation (EOBF) as a Disinfectant against African Swine Fever Virus (ASFV) agent in PAM cells of Swine. Introduction: The African Swine Fever Virus (ASFV) is considered one of the most pathogenic viruses of pigs. Results and Discussion: Antiviral activity against ASFV in PAM culture. Conclusion: The observed results demonstrated that EOB up to dilution ten could degenerate the lethal dose log 5 of ASFV.



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Use of an essential oil blend formulation (EOBF) as an effective disinfectant against pathogenic luminescent vibrio bacteria

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DOI: <https://doi.org/10.22271/fish.2021.v9.i2b.2455>

Abstract
Vibrio bacterial groups, mainly *Vibrio harveyi*, *V. campbellii*, *V. parahaemolyticus* and *V. splendidus*, are considered highly pathogenic to shrimp larvae. These bacteria become more aggressive and lethal in their luminescent form due to their quorum sensing characteristics. An effort was made to minimize the luminescent Vibrio load using an essential oil blend formulation (EOBF) consisting of *Eucalyptus* oil, *Jasmine* oil, and *Gardenia* oil in equal proportions. An eighteen-day small hatchery-scale trial was initiated, starting from the nauplii stage. The shrimp were distributed into three groups: a T-1 negative control with four replicates, a T-2 positive control with four replicates, and an EOBF treatment group with eight replicates, which was subgrouped equally into two rooms, named T-3 and T-4. The shrimp at the mysis stage were challenged using a sublethal dose of 10³ CFU/mL luminescent Vibrio.

Vibrio observations, physical properties of tank water and shrimp productivity were measured. The obtained results indicated that the EOBF-treated group had better productivity than the positive control group. The positive control T-2 performance at the time of harvest (PL 10 stage) was as follows: survival rate 67.57%, length 9.01 mm, size variation 12.62, mean body weight 4.51 mg and biomass 116.36 g. The performance of EOBF groups T-3 and T-4 at the time of harvest (PL 10 stage) was as follows: survival rate 56.34%, length 10 mm, size variation 10.21, mean body weight 7.71 mg and biomass 155.38 g. The EOBF group was better in terms of length, size variation, mean body weight and biomass. The presence of harmful Vibrio in tank water, recognized as green colonies cultured on TCBS agar, was almost one log lower in the EOBF group than in the positive control. The presence of pathogenic luminescent Vibrio was observed by the wet mount method, which showed that the incidence rate in the positive control (T-2) was 45%, whereas it was 19% in the EOBF group. The results of this trial suggest that EOBF can reduce pathogenic Vibrio in hatchery environments and can increase productivity.

Keywords: pathogenic vibrio, luminescent vibrio, *Vibrio parahaemolyticus*, *Vibrio harveyi*, essential oil blend formulation, disinfectant, shrimp hatchery, *Penaeus vannamei*

Introduction

Luminescent or glowing bacteria is the common terminology for harmful Vibrio bacteria in shrimp hatcheries. The colonies of this group of bacteria appear green on TCBS media. The most pathogenic species in the luminescent group is *Vibrio harveyi*, which is infectious to cultured shrimp, such as *Penaeus monodon* and *Penaeus vannamei* in hatcheries [1]. Apart from *Vibrio harveyi*, *V. campbellii* and *V. splendidus* can also produce luminescence and infect the larval, juvenile and adult stages of cultured shrimp [2, 3]. *Vibrio parahaemolyticus* has quorum factors that can stimulate luminescence in *Vibrio harveyi* quorum-sensing mutants [4], a result confirmed in this study. Some reports are available indicating that glowing *Vibrio harveyi* are able to infect juvenile shrimp in culture ponds [3]. Heavy mortality, even up to 100% in shrimp, can be caused by pathogenic Vibrio in combination with environmental stress [1, 5, 6, 7]. Luminescence is a source of communication in bacteria, making them more pathogenic and aggressive [8, 9].



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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].



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

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Abstract

The *Vibrio harveyi*, *V. campellii* and *V. parahaemolyticus* are considered highly pathogenic to shrimp larvae. Shrimp larvae are susceptible to Vibrio. It is challenging to eradicate Vibrio from the hatchery environment all over the world. There are no products available in the market to claim as a potential anti-Vibrio in market. Successful efforts were made to develop an effective disinfectant with primary ingredients of natural origins against pathogenic Vibrios in the shrimp hatchery. A formulation called Essential Oil Blend formulation and commercially known as Ecoguard was developed by blending eucalyptus, *Jasmine* and *Gardenia* oils with antimicrobial properties. The effective dose of Ecoguard was optimized by conducting multiple lab and commercial level trials on bacteria using TSA and on Vibrio using TCBS agar media. The optimized dose of 80 ppm Ecoguard has sprayed on the tank and culture area dry surfaces. The trial sites selected for this discussion 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 a freshwater tank of an experimental hatchery. The swab samples were collected before and after 3 hours of spray and a significant decrease in Vibrio, mostly green colonies such as *Vibrio harveyi* and *V. parahaemolyticus* were found after Ecoguard application (Table 1). The obtained results showed that EOBF is an effective disinfectant against pathogenic Vibrios in shrimp hatcheries.

Table 1: Comparative Vibrio reduction data on hatchery culture tank surfaces before and after Ecoguard spray.

Source	Before Ecoguard Application			Three hours after Ecoguard application		
	No of sample	No. of positive Vibrio	Vibrio (%)	No of sample	No. of positive 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 & 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	26	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
Polychaeta (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

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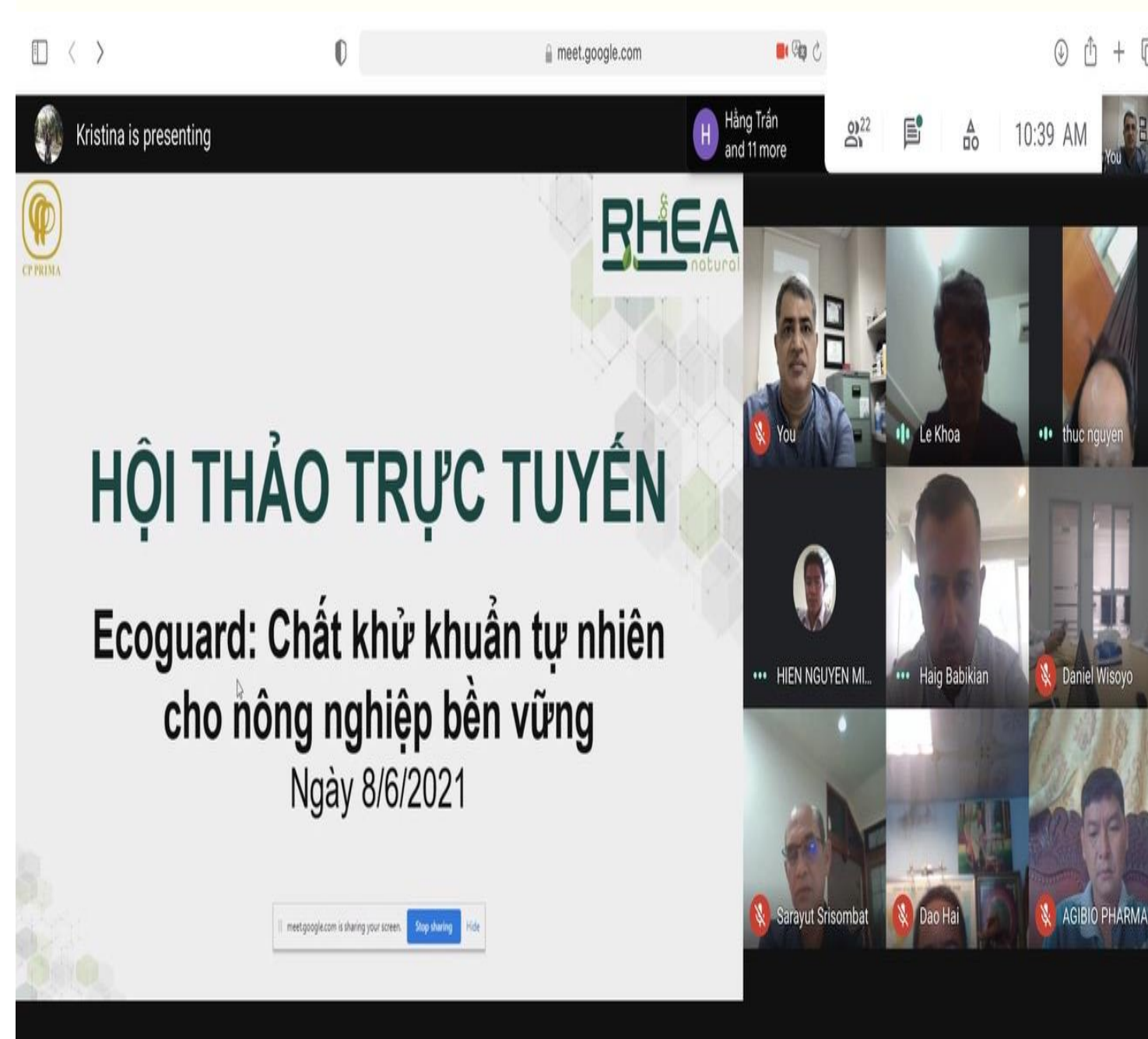
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A half-day workshop was successfully organized on June 8, with the help of the Hatchery, TRD, and Rhea teams. The workshop was focused on demonstrating Ecoguard dilution, spraying, and sampling collection to test E. coli, Salmonella (for pig farms and chicken farms), and Vibrio (for shrimp hatchery). The participants were limited to 20 people.

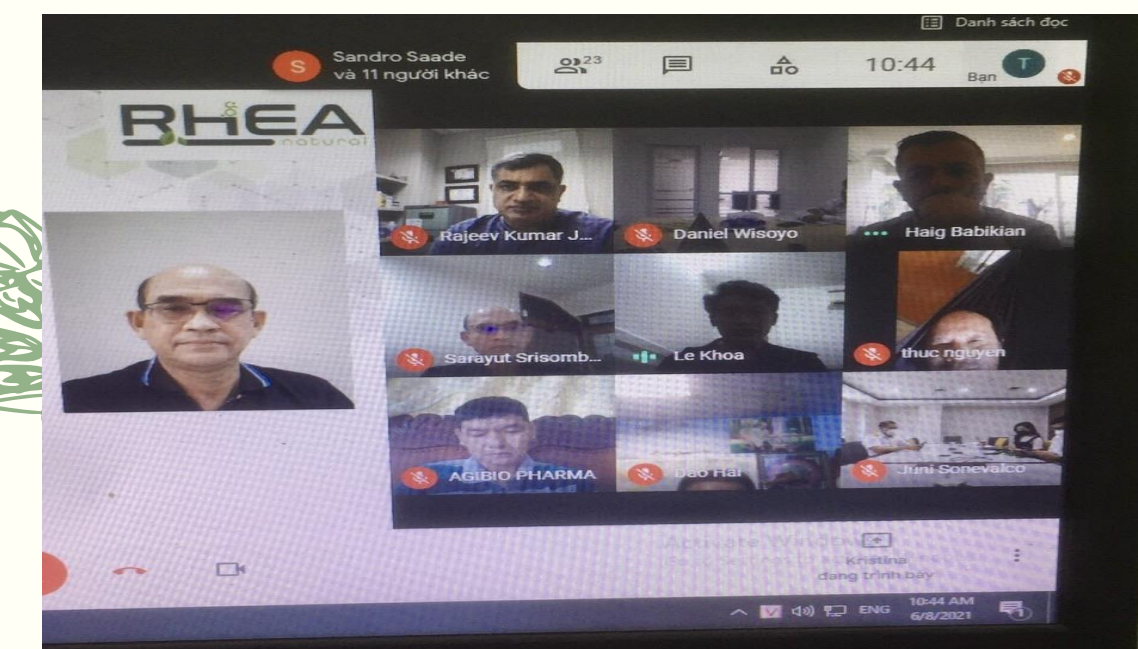
Workshop Agenda

Serial	Speaker	Topic	Time
1	Dr. Le Van Khoa and Ms. Kristina	Introduction of workshop, agenda and speakers	10.00-10.05
2	Prof. Yusef Babikyan	Opening remarks and welcome address	10.05-10.10
3.	Mr. Sarayut Srisombat Dr Haig Babikian	Brief Introduction of CP Prima and Rhea Natural Sciences	10.10-10.15
	Dr. Rajeev Kumar Dr. Le Van Khoa	Rhea Natural Sciences products introduction	10.15-10.30
Break: 5 minutes			
4.	Workshop 1 (Central Lab) Dr. Beni Halalludin	<ul style="list-style-type: none"> Ecoguard Preparation (precautions and biosafety) Media Preparation (<i>E. coli</i>, <i>Salmonella</i> and <i>Vibrio</i>) Identification of colonies Plate reading/colony counting Result interpretation 	10.35-11.20
Break: 10 minutes			
5.	Workshop 2 (Field demonstration) Mr. Rubianto Haliman Mr. Edi Poncolaksito Mr. Suiarwo	<ul style="list-style-type: none"> Ecoguard on site preparation Ecoguard Spraying procedure Ecoguard application Sampling procedure 	11.30-12.15
Break: 10 minutes			
6.	Prof. Yusef Babikyan, Mr. Sarayut Srisombat, Dr Haig Babikian, Dr. Rajeev Kumar, Dr. Le Van Khoa, Mr. Rubianto Haliman, Mr. Edi Poncolaksito	Panel Discussion and Q & A session	12.40-13.00



- Ecoguard hoạt động như chất khử trùng để loại bỏ hoạt tính và thoái hóa các mầm bệnh có trong bình xịt, dạng tự do, trên bề mặt sàn nhà, nhà kho và kho chứa.
- Có thể dùng như chất khử trùng để rửa các thiết bị.
- Không giống như các sản phẩm khử trùng khác thường độc hại, Ecoguard an toàn cho cả người và động vật.
- Ecoguard bao gồm chiết xuất từ các loại dầu tự nhiên: dầu hoa nhài, dầu cây dành dành và dầu bạch đàn.
- Mô tả sản phẩm: Chất lỏng trong suốt màu vàng, an toàn và thân thiện với người dùng.
- Bao bì: Chai thủy tinh 100 mL, 250 mL, 500 mL và 1L.
- Có thể áp dụng để khử trùng bằng phương pháp phun trong trại lợn, trại gà và trại sản xuất tôm giống

Application Method	Dose	Duration
Spraying	80 ppm	2-3 times a week



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