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PROJECT DELIVERABLE

[D 2.2.2] TEST RESULTS FROM THE LABORATORY SCALE TRIALS, RECOMMENDATIONS FOR FULL/LARGE SCALE TEST TRIALS

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This is a controlled document for any changes and amendments done for the deliverable.
Amendment shall be by whole document replacement.

Version	Status	Date	Authorized by
1	Draft version		
2	TCT and peer review		

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LIST OF ABBREVIATIONS / GLOSSARY

EU..... European Union

EC ..... European Community

CFU..... Colony Forming Unit

DEFT ..... Direct Epifluorescent Filter Technic

DOC ..... Dissolved Organic Carbon

EF ..... Effective Concentration

h..... Hours

HCl ..... Hydrochloric acid

H_2O_2 ..... Hydrogen peroxide

IMO..... International Maritime Organization

L..... Litre

MARTOB..... On Board Treatment of Ballast Water and Application of Low-sulphur Marine Fuel (EU research programme)

MEPC..... Marine Environment Protection Committee

mg ..... Milligramme

ml ..... Millilitre

m^3 ..... Cubic meter

ND ..... Not determined

NOAA ..... National Oceanic and Atmospheric Administration

NOEC ..... Non Observable Effect Concentration

nm ..... Nanometer

PAA ..... Peracetic Acid

PSU ..... Practical Salinity Unit

SEDNA ..... Safe Effective Deactivation of Non-indigenous Aliens

UV ..... Ultra Violet

WP ..... Work Package

µm ..... Micrometer

1 EXECUTIVE SUMMARY

The study of active substances for ballast water treatment was proposed regarding the state of the art of methodologies from scientific literature analyse and from previous research projects.

The first report (deliverable D2.2.1) included:

- The bibliographic analysis concerning any potentially usable active substances and their selection, based on three types of chemical compounds: peracetic acid, vitamin K3 and alkylamines.
- The bibliographic analysis concerning organisms living in ballast waters and the preliminary results from in situ sampling.

This second report gathers together all the results obtained after the first year of experimentations and analyses, including:

- A summary of the active substances and specifications.
- The results of their biocide effects, obtained from laboratory scale test trials.
- The results of their environmental acceptability, obtained from large scale trials.
- The presentation of the "ballastodrome" device and forward-looking experiments.
- The work progress concerning onboard tests.

The selected active substances are industrial products with different chemical bases: peracetic acid, vitamin K3 and alkylamines. The corresponding commercial products are respectively Peraclean® Ocean, SeaKleen® and Mexel®. Their efficiency was tested on bacteria, phytoplankton and zooplankton. The results showed that these biocides are efficient in different concentrations and that their effect varies regarding the bacteria strain and the organisms type (phyto- / zooplankton). Each active substance has a biocide effect, depending on the used concentration and the type of organism.

Concerning bacteria, peracetic acid and alkylamines seem to be the most effective biocides. Concerning their efficiency against phytoplankton (growth inhibition tests), all the products appear to be of a good efficiency when used at the concentration recommended by manufacturers. Finally, concerning zooplankton (*Artemia salina*), SeaKleen® was the most efficient of the three tested biocides in terms of the necessary concentration for reaching 90% mortality. All of these results seem to be in accordance with previous studies (bibliographic analysis). However, no previous data is available for the Mexel® product.

As the biocide efficiency tests have been conducted in the laboratory, the environmental acceptability study is a method to assess the added value of the products on large scale.

Environmental acceptability tests were performed with oyster larvae; they were carried out with several concentrations of the active substances. These ecotoxicological tests highlight that the alkylamine-based product (Mexel®) is the only tested biocide which displays no impact on oyster larvae development whatever the concentration.

The alkylamine-based product has been chosen for large scale experiments based on the ecotoxicological tests and the fact that this Mexel® product has never been used in ballast water treatment. The efficiency of the Mexel® biocide is tested through two different kinds of experiments in the ballastodrome system (test in progress): the first one uses an "EFFORTS soup" (mixture of bacteria, phytoplankton and zooplankton) and the second one uses natural populations of turbid seawater from the British Channel.

Finally, an onboard test will be performed with a ship-owner partnership, between April and June 2009.

2 INTRODUCTION

Regarding the European policy about ballast water treatment to prevent European coasts from invasive species, several methodologies and equipments have been proposed. Currently there are several ballast water treatment options available, which make use of active substances and which fulfil the IMO D-2 requirements. As the result of the IMO MEPC58 meeting held in London in October 2008, the final approval was given to following ballast water treatment options: the Electro-Cleen™ System (Republic of Korea), OceanSaver® Ballast Water Management (Norway), and NK-O3 BlueBallast System (Republic of Korea). Earlier the final approval has been granted to the SEDNA® using PERACLEAN® OCEAN Ballast Water Management System (Germany) and Alfa Laval's PureBallast (Sweden). Additionally, there are several systems that hold Basic Approval and most likely the manufacturers of those systems develop and test the systems to meet the requirements set for Final Approval.

The adopted solutions will probably be a combination of systems such as hydrocyclon or filtration (particle separator) associated with UV treatment, ozone or active substances.

Concerning active substances, the EFFORTS project is focused on three different chemical compounds: peracetic acid, vitamin K3 and alkylamines. These substances are available from manufacturers approved by international authorities and could be used for ballast water treatment. However, it is necessary to improve the knowledge about their efficiency and proprieties, including the ecotoxicologial aspect. The multidisciplinary group in the EFFORTS project (end-users, biologists, chemists, microbiologists, ecotoxicologists and corrosion experts) has chosen three scientific approaches: (1) assessment of substance efficiency through laboratory tests, (2) simulation with a bacteria, phytoplankton and zooplankton organisms soup in a half-open device and (3) onboard test to facilitate the end-users final choice.
A complementary ecotoxicological approach was also performed to assess the risks induced by deballasting of seawater with active substances in port environment. As the port environment is a sensitive area regarding environmental coastal impacts, this point is particularly important: the port area is the final recipient of treated ballast water.

3 OBJECTIVES

The investigations strive out towards the following main objectives:

- To characterize the main organisms from real ballast water and to isolate potential pathogen species.

- To define the ecotoxicological effect of three different types of active substances (organic acid, vitamin K and alkylamine) regarding their concentration and exposure time.

- To compare the efficiency of these active substances against bacteria, phytoplankton and zooplankton. Laboratory experiments allowed to determine the active substance to reach the best compromise between its biocide efficiency and its environmental acceptability. This active substance was selected for large scale tests.

- To develop a biological soup (mixture of bacteria, phytoplankton and zooplankton) associated with an experimental device named "ballastodrome". The result evaluate the efficiency of the active substance against a seawater type close to real conditions (simulator).

- To test, in situ, the active substance in operational onboard conditions of a commercial ship. This final step allows to evaluate the previous results (highlighted in the bibliographic analysis) and further to detect the main limits of this solution regarding onboard constraints and ship specifications.

- Finally, to prepare recommendations for ship owners and port authorities, regarding the potential impacts of active substances on port environments.

4 ACTIVE SUBSTANCES SELECTED

4.1 General

The first task in the WP 2.2.1 was to assess the efficiency of active substances against organisms for ballast water treatment. As the result of the literature survey three biocides were selected for the laboratory scale test trials. Two of the selected products, i.e. Peraclean® Ocean and SeaKleen®, were identified as potential biocides for ballast water treatment, since they were previously tested in laboratory and large/full scale test facilities. The third product, Mexel® 432/336, is a new candidate for ballast water treatment.
The more comprehensive information concerning the selected products and their previous test results has been presented in the deliverable Deliverable D2.2.1 of the EFFORTS-project.

4.2 Peracetic acid based biocide

Peracetic acid is a molecule with known strong oxidising properties. It denatures proteins, modifies cell membrane permeability and oxidises disulfide bonds of proteins and cellular metabolites. It displays a broad spectrum against micro-organisms ($Gram^+$ and $^-$ bacteria, Mycobacteria, viruses, fungi and spores) and a short action time.

The Degussa AG[1] has developed Peraclean® Ocean, which is a proprietary formulation based on peroxygen chemistry. The main components of the Peraclean® Ocean are peroxyacetic acid (PAA) (15%), hydrogen peroxide H_2O_2 (14.3 %) and acetic acid (26.5%). It has been demonstrated to effectively kill bacteria, yeasts, viruses, molds, spores, algae, protozoa, zooplankton and their larvae, phytoplankton larvae stages and fish eggs in ballast water. It is most effective when combined with separation of solids. Due to its unspecified mode of action as an oxidiser it is effective and no resistances have been observed so far. It is active at high concentrations of sediment and/or organic matter at pH values between 5 and 9 and at temperatures of -5 to more than 40 °C. It is a liquid that can easily be applied automatically with a proprietary technology.

The formulation has a long shelf life (> 95 % activity after one year of storage at 20 °C). The product is effective at relative low concentrations (100 ppm). The active ingredients of the product are neither bioaccumulative nor persistent, will ultimately degrade to oxygen, water and carbon dioxide and will not form any persistent metabolites. In ballast water residual Peraclean® Ocean decomposes to water, acetic acid (i.e. vinegar) and water (Dragsund et al., 2006; International Maritime Organisation (IMO), 2005).

The SEDNA® Ballast Water Management System, manufactured by the HAMANN AG (Germany), uses hydrocyclons and filter as pre-treatment and Peraclean® Ocean as Active Substance. The system has received both Basic Approval in February 2006 and Final Approval in April 2008, and it has been examined and tested according to requirements of the Guidelines for Approval of Ballast Water Management Systems (G8) adopted by IMO Resolution MEPC.125(53). The treatment rated capacity of the type approved system is 250 m^3 per hour, and the maximum dosage of Peraclean® Ocean is 150 mg per litre. Minimum retention time of treated ballast water is 24 hours (IMO, 2008b).

4.3 Vitamin K_3 based biocide

Description of K vitamins

Vitamin K is a family of structurally similar fat-soluble 2-methyl- 1,4-naphthoquinones including phylloquinone (K_1), menaquinones (K_2), and menadione (K_3). 1,4-naphthoquinones form a family of compounds characterized by a naphthalene ring containing two carbonyl moieties at positions 1 and 4, which in the case of vitamin K is substituted at positions 2 and 3. All members of the vitamin K family possess the identical napthoquinone skeleton with various side chains that distinguish them.

[1] As of September 12, 2007, Degussa is the Chemical Business Area of the Evonik Industries.

The best-known member of the vitamin K family is phylloquinone, also known as phytonadione or menaphthone, so named because of its intimate relationship with photosynthesis in plant leaves. Vitamin K is a cofactor for an enzyme that catalyzes the carboxylation of the amino acid, glutamic acid, resulting in its conversion to gamma-carboxyglutamic acid (Gla) (Furie *et al.*, 1999). Although vitamin K-dependent gamma-carboxylation occurs only on specific glutamic acid residues in a small number of vitamin K-dependent proteins, it is critical to the calcium-binding function of those proteins (Ferland, 2006; Shearer, 1997). Moreover vitamin K is essential for the functioning of several proteins involved in blood clotting (Brody T, 1999; (Lamson *et al*., 2003).

Vitamin K_3 (menadione; 2-methyl-1,4-naphthoquinone) is a polycyclic aromatic ketone, based on 1,4-naphthoquinone, with a 2-methyl group (Figure 4.3) and is the precursor to various type of vitamin K. For example, after ingestion vitamin K_3 is converted into the active form of vitamin K_2 in the liver. Hence it is better classified as a provitamin (Innopharmchem.com, 2009).

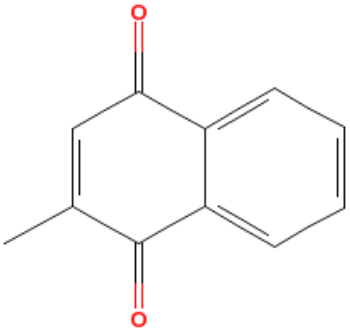


FIGURE 4.3. CHEMICAL STRUCTURE OF K_3 VITAMIN OR MENADIONE.

Vitamin K_1 and K_2 are the naturally occurring types of vitamin K. The former is synthesized by higher plants as well as algae and can be found in such foods as spinach, broccoli, lettuce, and soybeans. The latter, sometimes alternatively referred to as menaquinone, is primarily produced by bacteria in the anterior part of the gut and the intestines. Vitamin K_3, on the other hand, is not naturally found but exists in synthetic form (yellow crystalline substance). Vitamins K_1 and K_2 are metabolized through the lymphatic system, utilizing pancreatic enzymes and bile acids and regulated by the liver. Vitamin K_3 is absorbed directly and bypasses the natural pathways and regulators.

Toxicity of K_3 vitamin

Although allergic reaction is possible, there is no known toxicity associated with high doses of vitamin K_1 or vitamin K_2 (Food and nutrition board, 2001) and a vitamin K deficiency can be dangerous especially to infants that may easily suffer from extensive haemorrhaging. On the other hand, menadione given by injection has induced liver toxicity, jaundice, and hemolytic anemia (due to the rupture of red blood cells) in infants. Therefore, menadione is no longer used for treatment of vitamin K deficiency (Olson, 1998; Ferland, 2006).

Vitamin K_3 can interfere with the function of glutathione, one of the body's natural antioxidants, resulting in oxidative damage to cell membranes. Additionally the production of potent singlet oxygen in quantities slightly greater than normal destroys pigments like chlorophyll in plants. More sophisticated theories that do not fit into the oxidative model have been proposed to explain puzzling aspects of the anticancer effects of vitamin K_1 and K_2 versus K_3, such as the induction of apoptosis, differentiation, and cell cycle inhibition (Lamson *et al*., 2003).

SeaKleen® is based on K_3 vitamin and was developed in part through the NOAA[2]-supported laboratory program in the US and was patented for control of pests in ships' ballast water (US Patent #6,340,468, Cutler *et al*., 2004). It is delivered as a soluble powder which is dissolved in water prior to being injected into the influent ballast water stream. Concerning the handling of SeaKleen® no special training is needed (Wright, 2004).

4.4 Alkylamines based biocide

Akylamines named equally fatty amines are a family of molecules containing at least one hydrocarbon chain (lipophilic) and one nitrogen group (hydrophilic). These amphiphilic substances have surface actives properties inducing filming properties. The alkylamines are classified as non-oxidising biocides by the IPPC (2000). In water circuits, the adsorption of the alkylamines on inorganic surfaces such as metals or suspended solids provide corrosion inhibitor properties and reduce the mud deposits. Equally, towards bio-fouling, their integration in the biological membranes induces biocidal activities. That is why, according to Toxicology/Regulatory Services, Inc. (2003), such substances are known to kill aquatic organisms via surfactants activities rather than chemical mechanism.

Mexel® 432/336 is an emulsion of alkylamines in an aqueous phase. This new product is in accordance with the European Biocide Directive 98/8/CE (European Parliament and Council of February 16, 1998). The Mexel® product contains no halogens, aromatics, quaternary amines, heavy metals, sulphur, cyclic hydrocarbons, zinc or oxidizing agents and consequently is environmentally acceptable. Its biodegradation only produces carbon dioxide, water and a trace of nitrogen and it degrades quickly (within one or two days) (Chattopadhyay *et al.*, 2004). The Mexel® product acts as a detergent which displays anticorrosive and antifouling properties. Moreover, it acts on biofilms and against calcareous deposit and has liquefying effects on sludge. Finally the non-mutagen effect of Mexel® has been proved (Ames test (Ames et al., 1975)) and it is safe to use.

Although Mexel® 432/336 appears to have many advantages in terms of biological efficiency against aquatic organisms and bacteria, its potential as a biocide for ballast water treatment has not been clearly established.

[2] National Oceanic and Atmospheric Administration (NOAA).

5 BIOCIDE EFFECTS FROM LABORATORY SCALE TRIALS

5.1 *In situ* sampling procedure of organisms

Seven ballast waters samplings were collected directly in ballast tanks from several ships (containers and bulk carriers). the sampling campaigns were conducted in three French ports: La Rochelle-Pallice, Rochefort-Tonnay Charente and in the autonomous port of Le Havre. Details of the campaigns are summarized in Table 5-1.

According to the organisms further studied, 1 to 3 litres of ballast waters and 1m^3 of 80 µm filtered water were taken (see report D2.2.1. for sampling methodologies) (Pineau S. et al., 2008)).

TABLE 5.1 DATA ON SAMPLING CAMPAIGNS

Port	Date of sampling	Type of ships	Sampling procedure	Ballast tank localization	Origin of ballast waters
La Rochelle - Pallice	November 2007	Bulk carriers	Overflow on the deck	Afterpeak	NETHERLANDS (mixed with La Rochelle)
Rohefort-Tonnay Charente	December 2007	Bulk carriers	Overflow on the deck	Forepeak	PORTUGAL
Le Havre	December 2007	Container carriers	Manhole	Double bottom tanks	SOUTH-EAST ASIA
Rohefort-Tonnay Charente	December 2007	Bulk carriers	Overflow on the deck	Double bottom tanks	Pasajes, SPAIN
Le Havre	January 2008	Container carriers	Checking valve of ballast pump	Double bottom tanks	CHINA
Le Havre	January 2008	Container carriers	Manhole	N°5 UP, "Central side" of the boat	Mix mid-Atlantique Ocean / Anvers
Le Havre	January 2008	Container carriers	Ballast valve	Double bottom tanks	Water exchanged in Atlantic Ocean (The Bermuda)

These samples were then analysed for their qualitative and quantitative content in bacteria, phytoplankton and zooplankton species.

5.2 Organisms identification and numeration

5.2.1 Results of bacteria analysis

The bacteriological investigation focused on five genera which have been chosen considering their potential human and/or animal pathogenicity and the IMO regulations. These are *Vibrio*, *Aeromonas*, *Pseudomonas*, fecal enterococci and coliforms.

First, the number of total viable and cultivable bacteria was determined by counting the colonies formed on marine agar plates (Scharlau Chemie S.A). This media was specifically formulated for heterotrophic marine bacteria growth (ZoBell, 1941). It contains minerals that mimic the major mineral composition of seawater and a high level of salts to simulate seawater.
Second, bacterial strains were isolated from ballast water samples using selective media and then identified by biochemical tests.
Methodologies of these two experiments are described in the previous report D2.2.1 (Pineau S. et al., 2008).

Third, on the last two samples, a total bacteria (i.e. live and dead bacteria) count was performed by the Direct Epifluorescent Filter Technic (DEFT). Collected samples (5 ml) were stained with acridine orange (500 µl) for 5 minutes in dark at room temperature. The mixture was then filtered on a 0,2 µm polycarbonate black membrane (*Nucleopore*®) and bacteria were observed under the microscope (*Olympus*® BH2-RFCA). Indicated values are a mean of 20 counted fields which are randomly distributed on the membrane.

Results obtained on each sample are listed in Table 5-2-1.

As a result, about 60 colonies grown on selective media were isolated, cultured and biochemically tested. 12 of them were clearly identified and stored. Ballast water originating from Northern Spain, South-east Asia and Atlantic Ocean showed the highest concentration of cultivable bacteria (10^3 to 10^4 CFU/ml). This suggests that the current procedure which consists in exchanging ballast waters at high seas (Ballast water Exchange standard, IMO 2004) might not be proper and sufficient to eliminate bacteria from ballast tanks. However these levels of contamination are consistent with previous studies (Mimura *et al.*, 2005; Ramaiah *et al.*, 2005). Finally, the counting of total bacteria showed that less than 1% of marine bacteria are viable and cultivable which corroborates previous observations (Jannash and Jones, 1959).

TABLE 5.2.1 RESULTS OF THE BACTERIA ANALYSIS ON THE SEVEN SAMPLES.

ORIGIN OF BALLAST WATER	IDENTIFIED BACTERIA	TOTAL VIABLE AND CULTIVABLE BACTERIA	TOTAL BACTERIA
NETHERLANDS (mixed with La Rochelle)	*Aeromonas caviae, Vibrio parahaemolyticus, Pseudomonas fluorescens*. No Enterococci	35 CFU/ml	nd
PORTUGAL	*Aeromonas salmonicida salmonicida, Enterococcus faecalis, Citrobacter freundii*	305 CFU/ml	nd
SOUTH-EAST ASIA	*Vibrio alginolyticus, Vibrio fluvialis, Enterococcus faecalis*. No coliforms	$3,1.10^3$ CFU/ml	nd
Pasajes, SPAIN	*Pseudomonas fluorescens*. No Vibrios, no Enterococci, no coliforms.	$1,6.10^4$ CFU/ml	nd
CHINA	*Shewanella putrefaciens, Pseudomonas stutzeri.* No Enterococci, no coliforms.	No colony on marine agar	nd
Mid-Atlantique Ocean / Anvers mixed waters	*Vibrio vulnificus, Aeromonas caviae, Enterococcus faecalis, Escherichia coli, Pseudomonas fluorescens*.	No colony on marine agar	$1,3.10^6$ bacteria/ml
Water exchanged in Atlantic Ocean (The Bermuda)	*Pseudomonas stutzeri*. No Enterococci, no coliforms	$1,2.10^3$ CFU/ml	$7,8.10^5$ bacteria/ml

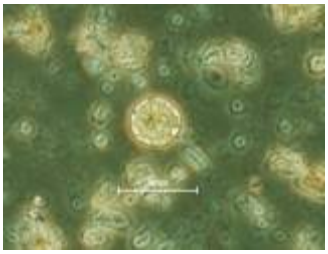
nd: not determined

5.2.2 Results of phytoplankton analysis

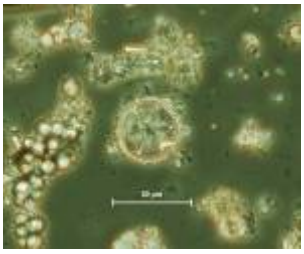
The Genus *Pseudonitzschia*, observed in two samples, is potentially harmful.

TABLE 5.2.2 RESULTS OF THE PHYTOPLANKTON ANALYSIS ON THE SEVEN SAMPLES.

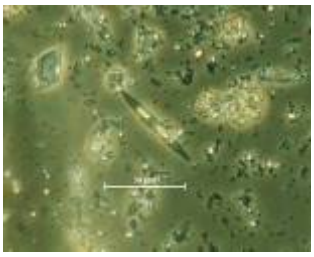
ORIGIN OF BALLAST WATER	Taxon	Nb of cells/ml
NETHERLANDS (mixed with La Rochelle)	*Coscinodiscus* *Actinodiscus*	400 300
PORTUGAL	*Actinodiscus* *Pseudonitzschia*	300 200
SOUTH-EAST ASIA	None	
Pasajes, SPAIN	None	
CHINA	None	
Mid-Atlantique Ocean / Anvers mixed waters	*N. longissima* *Thalassiosira* *Navicula* *P. marina* *Pseudonitzschia*	500 200 200 1300 100
Water exchanged in Atlantic Ocean (The Bermuda)	*Ditylum*	100



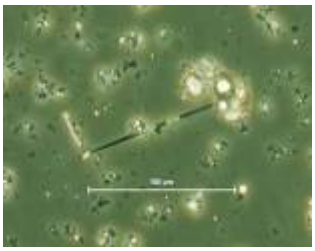
Coscinodiscus (Diatomophyceae)



Actinodiscus (Diatomophyceae)



Pseudonitzschia (Diatomophyceae)



Pseudonitzschia (Diatomophyceae)

5.2.3 Results of zooplankton analysis

Zooplankton taxa were analysed from ballast water samples originating from three different areas. In these samples, the most abundant group were copepods, including cyclopoids, harpacticoids and coastal calanoid species such as *Acartia* sp. and *Centropages* sp. (Table 5-2-3). The second abundant group was barnacle larvae. In addition to copepods and barnacle nauplii, low numbers of rotifers (*Keratella* sp.) as well as two specimens of resting eggs of daphnid cladocerans were observed (Table 5-2-3).

TABLE 5.2.3 ABUNDANCES OF ZOOPLANKTON OBSERVED IN BALLAST WATER SAMPLES ORIGINATING FROM DIFFERENT AREAS.

ORIGIN OF BALLAST WATER	TAXONOMIC GROUP	ABUNDANCE (ind/m^3)
Amsterdam / La Rochelle mixed waters	Calanoid nauplii	4
	Cyclopoid adults and juveniles	10
	Cyclopoid nauplii	1
	Harpacticoid adults and juveniles	10
	Copepoda sp. nauplii	2
	Barnacle nauplii	21
	Keratella sp.	4
	Daphnid resting eggs	1
	TOTAL	**53**
Portugal	Calanoid adults and juveniles	8
	Calanoid nauplii	29
	Cyclopoid adults and juveniles	7
	Cyclopoid nauplii	44
	Harpacticoid adults and juveniles	35
	Harpacticoid nauplii	1
	Copepoda sp. adults and juveniles	1
	Copepoda sp. nauplii	3
	Barnacle nauplii	46
	Keratella sp.	2
	Daphnid resting eggs	1
	TOTAL	**177**
China	Cyclopoid adults and juveniles	2
	Harpacticoid adults and juveniles	9
	Copepoda sp. adults and juveniles	1
	Barnacle nauplii	12
	TOTAL	**24**

5.3 Experimental processes for biocide effect assessment

Biocide assays were performed on 3 bacteria species isolated from the sample campaigns (*Vibrio vulnificus*, *Aeromonas caviae* and *Enterococcus faecalis*). In addition 2 phytoplanktonic species (*Alexandrium minutum*, *Tetraselmis suecica*) and 1 zooplanktonic genus (*Artemia*) originated from strains collections were tested.

The objective of these laboratory tests was to determine the most efficient biocide for each organism, i.e. the substance which yields 90% of lethality (LC90) (MEPC126(53), 2005) or growth inhibition after 24 hours ("effect") at a concentration which induces no "after-effect" and/or no ecotoxicological effect. In the case of bacteria and zooplankton two salinities were tested: 10 and 30 PSU which correspond respectively to brackish water and seawater.

For phytoplankton only 30 PSU was used because 10 PSU appeared unusable for marine phytoplankton. "Effect" assays were incubated 24 hours in darkness at 17°C to mimic the real ballast tank conditions (Gregg and Hallegraeff, 2007). The "after-effect" consisted in testing the environmental acceptability of the treated seawater after 24 hours, i.e. the half-life time period indicated by manufacturers for each biocide. Global procedure for this experiment was as follow: after 24 hours of incubation at 17°C in darkness, remaining organisms were removed from the treated seawater by filtration and then incubated again with a new batch of organisms, in a similar manner and using the same organism concentrations as in the "effect" test. The survival of organisms was then checked (Figure 5.3). This experiment helps to define if the remaining biocide concentration after 24h exposure is acceptable for the tested organisms and was performed for zooplankton and bacteria species. Regarding phytoplankton the numerous cell counts take a lot of time; subsequently the choice led to limit the number of experiments, particularly for the "after-effect" assessment. Instead, the environmental acceptability of the treated waters was assessed by oyster larvae bioassays (see section 5.3.4 for detailed procedures). "Effect" and "after-effect" experiments were reproduced 3 to 5 times depending on the studied organism.
Experimental parameters for biocide tests were accurately defined for all organisms and are listed in Table 5-3. Lab tests procedures were validated by a reviewer, Professor Erkki Leppäkoski from Åbo Akademi University, Finland.

TABLE 5.3 EXPERIMENTAL PARAMETERS FOR BIOCIDE ASSAYS: "EFFECT" AND "AFTER-EFFECT".

	Seawater	Volumes	Organism initial concentration *	Salinity of seawater	T°C	Light conditions	Biocide "effect" measurement	Replicates
Bacteria	Filtered natural seawater from the English channel	0,01L	10^5 cells/ml	10 and 30 PSU	17°C	Darkness	LC90 after 24h exposure	2 or 3
Phyto-plankton	Filtered natural seawater from the Atlantic Ocean	10L	10^6 cells/L	10 and 30 PSU	17°C	Darkness	Growth inhibition	3
Zoo-plankton	Filtered natural seawater from the Baltic Sea	9L	20 individuals/L	10 and 30 PSU (adjusted with "Meersalz aquarium salt)	17°C	Darkness	LC90 after 24h exposure	3

[1]Regarding the various origins of seawater, pH and DOC (Dissolved Organic Carbon) parameters were measured in each case and checked for their consistency among themselves and with IMO standards.

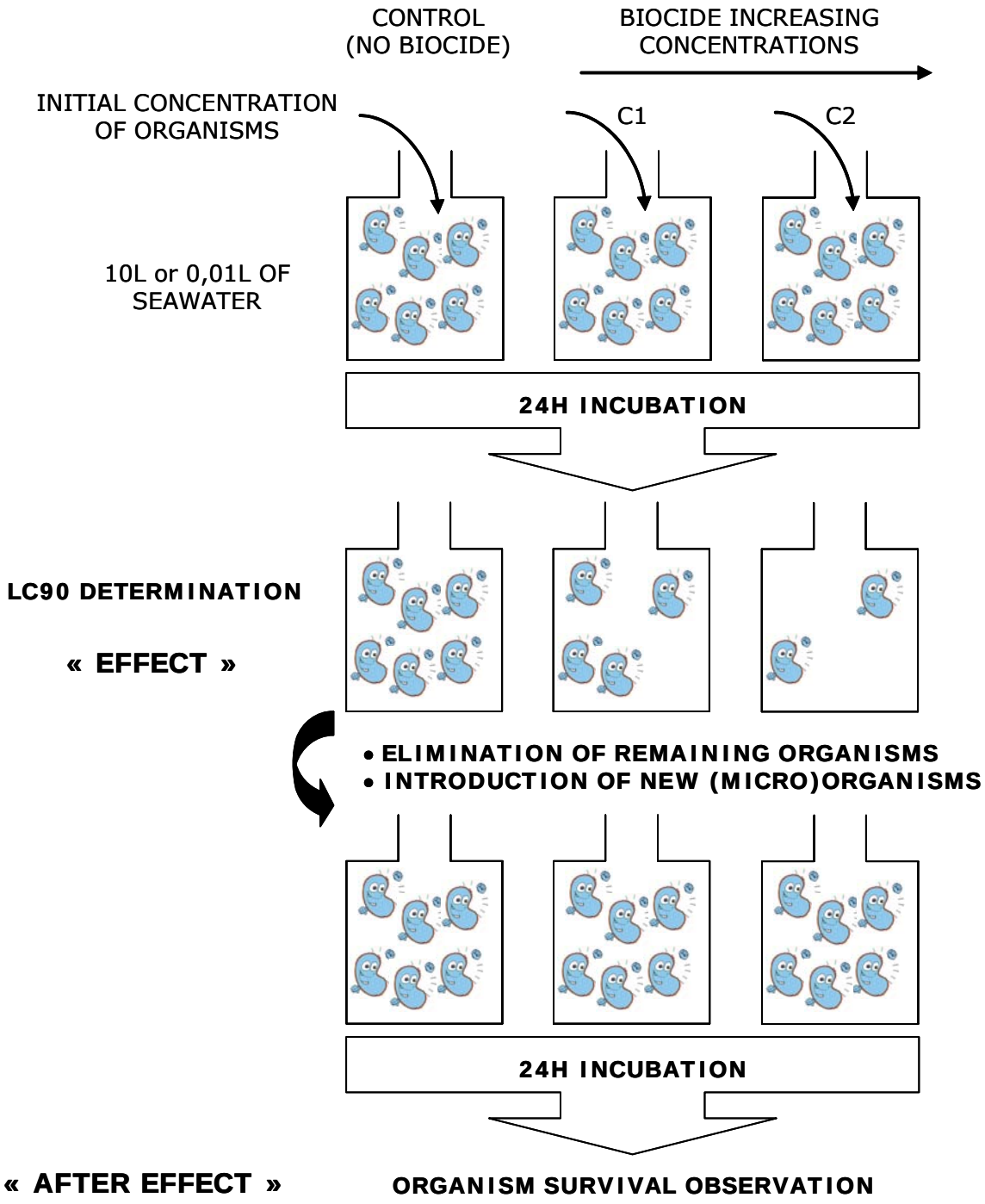


FIGURE 5.3 EXPERIMENT PROCEDURE.
C1 and C2 correspond to tested concentration 1 and tested concentration 2.

5.3.1 Methods for "effect" and "after-effect" assays on bacteria

Peraclean® Ocean, Mexel® and Seakleen® were separately tested on three bacterial strains isolated from the sample campaigns: *Vibrio vulnificus*, *Aeromonas caviae* and *Enterococcus faecalis*. *Vibrio vulnificus* is a gram negative bacterium responsible for sepsis and wound infections in humans and for "red pestis" in eel which induces important economical losses for eel farms. Another gram negative bacterium is *Aeromonas caviae* which is of concern as a food borne illness and is associated with gastrointestinal disease and chronic diarrhoea in humans. Finally *Enterococcus faecalis* which is gram positive being a natural member of the digestive microflora of humans and other animals and therefore it is a faecal contamination indicator. Moreover it is an opportunistic pathogen which is responsible for 80 to 90 % of nosocomial infections caused by Enterococci.

When mentioned cell concentrations were assessed by inoculating appropriate dilutions (in sterile physiological water, NaCl 9 g/L) in mass in nutritive agar (Biokar Diagnostics). After 48 hours of incubation at 30°C developed colonies were counted and the cell concentration was defined as colony-forming unit (CFU) / ml.

For "effect" determination an overnight culture of each bacteria strain was added at 10^5 cells/ml (Table 5.3) in separate container of 250 ml of filtered seawater collected in Luc sur mer (FRANCE, English channel) and adjusted to 10 or 30 PSU. The English channel was chosen for practical reason (close to our laboratory). Initial cell concentration (t_0) was checked and 10 ml were transferred in tubes before addition or not (control) of biocides at various concentrations (Table 5.3.1). Then tubes were incubated in darkness at 17°C for 24 hours. The cell concentration after 24 hours of incubation was determined and the survival percent was calculated relative to the cell concentration at t_0 in order to define the concentration of biocide which yields to 90 % of mortality (LC90).
Subsequent to these 24 hours of incubation the treated seawater was filtered through a 0,2 µm membrane (cellulose acetate, VWR) to eliminate all bacteria and inoculate again with 10^5 cells/ml of each bacteria. After further 24 hours of incubation at 17°C in darkness the "after-effect" of each biocide was assessed via survival percent as for "effect" experiment. Each set of experiments was independently reproduced 2 or 3 times. For each biocide, concentrations in the water prior to and after 24 hours of incubation were determined.

TABLE 5.3.1 BIOCIDE CONCENTRATIONS USED IN THE BIOASSAYS WITH BACTERIA.

	CONCENTRATION 1	CONCENTRATION 2	CONCENTRATION 3	CONCENTRATION 4
PERACLEAN® OCEAN	0,5 mg/L	5 mg/L	20 mg/L	
SEAKLEEN®	100 mg/L	200 mg/L	500 mg/L	
MEXEL®	2 mg/L	10 mg/L	20 mg/L	50 mg/L

5.3.2 Experimental procedure for «effect» assays on phytoplankton

Due to non-sufficient phytoplankton collected aboard ships, cultivated species were used instead. *Tetraselmis suecica* (Chlorophyceae) was chosen as a very resistant species (even surviving on tiled floor) and very often used by ballast water treatment system designers for efficiency assessment. Moreover it was also used for safety reasons as non toxin-producing, because it is cultivated in a shellfish hatchery. A second batch of experiments was carried out with the toxin–producing species *Alexandrium minutum*. This latter species needs a specialised secure laboratory in another location.

The biocide efficiency was measured by growth inhibition (Arzul *et al.*, 1999). This test was used instead of measurements of LC90 which were initially planned, but which requires vegetative forms or cysts for a viability assessment, fluorescence techniques, and more resources than available.
The development of a monospecific culture of phytoplankton in 150 ml of 0,2 µm filtered seawater (30 PSU) at 17°c with the selected active substances of various concentrations was followed during 4 or 5 days. Depending on the species a culture the medium was added to the seawater: Varicon-aqua (Cell-HI medium) for *Tetraselmis suecica* and L1 medium for *Alexandrium minutum* (Guillard *et al.*, 1993).
In total 5 ml of daily samples were fixed by Lugol solution and cell concentrations (number of cells/µl) were determined by counting with a Malassez cell, including live and dead cells. At day 0, one biocide was added at a given concentration (Table 5.3.2) and inhibition of growth was measured by the percent of cells in the treated assay compared to the control (i.e. 100 % of cells in the control at each time). The growth of the control samples was followed and expressed in number of cells per litre. Each condition was performed in triplicates.

TABLE 5.3.2 BIOCIDE CONCENTRATIONS USED IN THE "EFFECT" ASSAYS WITH PHYTOPLANKTON.

	BIOCIDE CONCENTRATIONS
PERACLEAN® OCEAN	100 mg/L – 10 mg/L– 0,2 mg/L – 0,1 mg/L
SEAKLEEN®	2 mg/L - 0,2 mg/L
MEXEL®	50 mg/L - 5 mg/L – 0,05 mg/L – 0,02 mg/L – 0,01 mg/L

5.3.3 Methods for «effect» and "after-effect" assays on zooplankton

Bioassays with zooplankton were carried out with the brine shrimp *Artemia* sp., owing to its ease of culturing and extensive use as a test species in various toxicity tests (e.g. Nunes *et al.*, 2006). In addition, a wide tolerance to various salinities make *Artemia* an excellent test organism.

The test medium was 0.2 µm filtered natural seawater collected from the northern Baltic Sea, from a port area of the Helsinki city. Due to the low salinity of the northern Baltic waters (4–6 PSU), hw-Meersalz aquarium salt was added to adjust the salinity to 10 or 30 PSU.

Artemia nauplii were obtained by hatching from resting eggs under optimal conditions (25–28°C, salinity 10 or 30 PSU, depending on the test trial).

One to four days old naupliar stages (instars II–V) were used in the experiments. At the start of a bioassay, *Artemia* density in the culture was determined, and an appropriate volume of the culture was gently poured on a 100-µm sieve and rinsed to the buckets to produce an experimental density of ~20 individuals/L. The appropriate biocide concentrations were prepared from stock solutions according to manufacturers' instructions. Five concentrations were tested for Mexel®, while for PeraClean® Ocean and SeaKleen® three concentrations were tested (Table 5.3.3).
Three replicates were run for each concentration. For Mexel® and PeraClean® Ocean, biocide concentrations in the water prior to and after the tests were determined with rapid concentration measurement kits supplied by the manufacturers.

TABLE 5.3.3 BIOCIDE CONCENTRATIONS USED IN THE BIOASSAYS WITH ARTEMIA.

Biocide	Biocide concentrations (mg/L)				
	1	2	3	4	5
Peraclean® Ocean	200	400	800		
SeaKleen®	1	4	8		
Mexel®	3	5	10	20	50

After the incubation period of 24 h, *Artemia* were gently collected from the buckets by pouring the contents through a 100-µm sieve, transferred to fresh 0.2 µm filtered seawater and examined within 2 h under a stereomicroscope. Live and dead individuals were counted separately from unpreserved samples. Inspection of unpreserved samples also allowed counting separately still living but injured individuals. Judgement between fit and injured individuals was done visually based on swimming behaviour.

In addition to the "after-effect" test, degradation after a longer retention time (7–28 days) was tested. The biocide-water solution used in the "effect" and "after-effect" incubations was stored in darkness (at 17°C), after which residual toxicity was examined by running a bioassay in a similar manner as in the "effect" and "after-effect" tests, using the same organism abundance (20 ind./L) and a 24-h incubation period.

Mortality rates were determined by dividing the number of dead individuals by the total number of individuals in each replicate, and the mean mortality in the control was subtracted from the obtained mortality rates in the biocide treatments. Logistic regression was fitted to the mortality data to determine LC50 and LC90 in 24 h.

Finally water properties were determined. At the beginning of the trials, samples for DOC determination were collected from the water to be treated with a syringe and preserved with 0.2 M HCl until analysed, and salinity, pH and temperature were measured with a portable YSI pH meter. Temperature in the bioassays was 16.4±0.5°C (mean ± SD). Salinity was slightly lower than intended, being 9.4±0.2 PSU and 25.4±0.7 PSU in the low and high salinity treatments, respectively, and the corresponding pH values were 8.3±0.2 (low salinity) and 8.6±0.2 (high salinity). For consistency, treatments with the two different salinities will be hereafter referred to as 10 PSU and 30 PSU treatments. Dissolved organic carbon (DOC) content in the water was on average 12.2±3.1 mg/L, which is well above the level required in the MEPC guidelines for the salinity range used (>5 mg/L; MEPC 2005).

5.3.4 Methods for ecotoxicological tests on oyster larvae

A marine ecosystem is generally considered in good health when organisms can reproduce normally. A good assessment is the use of ecotoxicologic tests on *Crassostrea gigas* oyster larvae (His *et al*., 1993).

Oyster broodstocks conveniently conditioned in hatchery were induced to spawn by thermal shock (28°C then 18°C alternatively). This way to induce spawning is more natural than stripping. One oyster broodstock couple was then chosen for fertilisation in vitro. Resulting in larvae were incubated 24 hours with the biocide at a given concentration (see Table 5.3.4) in 20 ml of filtered seawater at 24°C (Figure 5.3.4). Then at a specified D-stage (whose name is linked to the normal larvae form), larvae were fixed by formaldehyde and abnormal larvae were counted (i.e. no D-form). Each experiment was reproduced 5 times on 600 individuals.
The percentage of abnormal larvae was then compared to the control (i.e. incubation without biocide) and environmental acceptability level of the biocide was estimated using the following table (His *et al*., 1993):

- <5%: no effect,
- < 25%: mild effect,
- 25 to 50%: average effect,
- 50 to 75%: very effective effect,
- >75%: unacceptable effect.

TABLE 5.3.4 BIOCIDE CONCENTRATIONS USED IN ECOTOXICOLOGICAL TESTS WITH OYSTER LARVAE.

	BIOCIDE CONCENTRATIONS
PERACLEAN® OCEAN	200 – 100 – 50 – 20 – 10 – 5 – 1 - 0,1 – 0,001 mg/L
SEAKLEEN®	0,2 - 0,1 - 0,01- 0,005 mg/L
MEXEL®	50 – 20 – 10 – 5 – 3 - 0,1 - 0,05 - 0,01 mg/L; 50 – 20 -10 mg/L 2 to 3 weeks old

FIGURE 5.3.4 MALE AND FEMALE OYSTERS SPAWNING WITH 20 ML VIALS FOR LARVAE INCUBATION.

5.4 Efficiency against bacteria

The results showed that biocides are efficient in different concentration ranges and that their effect varies regarding the strain and the salinity (Figure 5.4.1, 5.4.2 and 5.4.3).

5.4.1 PeraClean® Ocean assay

Efficient concentrations to reach LC90 varied between 0,5 and 20 mg/L (Figure 5.4.1 and Table 5.4.1) depending on the salinity of the seawater. Indeed in 10 PSU seawater treated with 20 mg/L of PeraClean® Ocean, no bacterium survives.
The most sensitive strain is *Aeromonas caviae* (0,5<LC90<5 mg/L) and the most resistant one is *Enterococcus faecalis* (LC90=5 to 20 mg/L). Moreover salinity seems to influence the survival of *E. faecalis* and *V. vulnificus*. Indeed in 30 PSU seawater 20 mg/L of PeraClean® Ocean is required to eliminate 90% of *E. faecalis* compared with only 5 mg/L in 10 PSU seawater. On the contrary for *V. vulnificus* LC90 is attained at 0,5 mg/L in 30 PSU seawater whereas a concentration between 0,5 and 5 mg/L is needed in 10 PSU seawater.

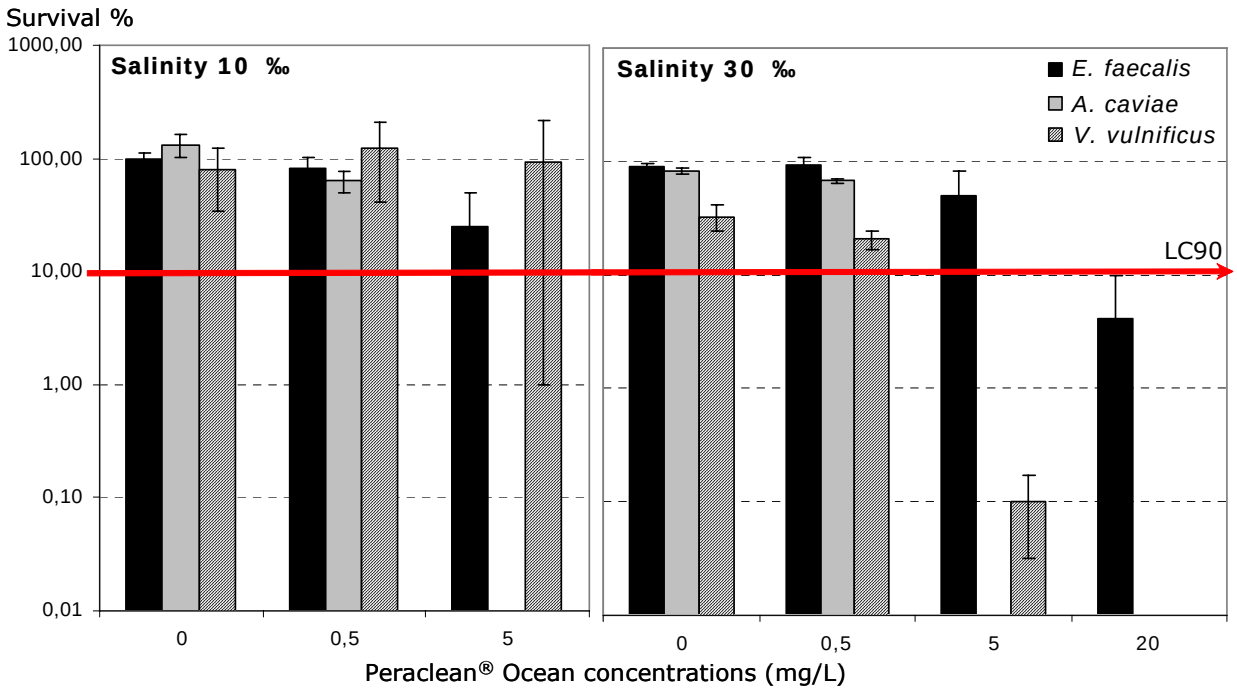


FIGURE 5.4.1 «EFFECT» OF PERACLEAN® OCEAN ON *Enterococcus faecalis*, *Aeromonas caviae* AND *Vibrio vulnificus*. Incubation was performed for 24 hours at 17°C in darkness in 10 PSU and 30 PSU filtered seawater. *Survival percent are mean values of three independent experiments and standard deviations are indicated*.

TABLE 5.4.1 LC90 OF PERACLEAN® OCEAN.

	10 PSU seawater	**30 PSU seawater**
Enterococcus faecalis	5 mg/L	20 mg/L
Aeromonas caviae	0,5 < LC90 < 5 mg/L	0,5 < LC90 < 5 mg/L
Vibrio vulnificus	0,5 < LC90 < 5 mg/L	0,5 mg/L

5.4.2 SeaKleen® assay

Regarding SeaKleen® *E. faecalis* and *V. vulnificus* have identical behaviours: 90% of bacteria are dead at 100 mg/L of SeaKleen® for both salinities used (Figure 5.4.2 and Table 5.4.2). However survival of *A. caviae* is not affected by 500 mg/L of SeaKleen® (even 1000 mg/L, data not shown) in 10 PSU seawater but this concentration yields 90% mortality (LC90) in 30 PSU seawater. Longer incubation time (48h) has no impact on these last results (data not shown).

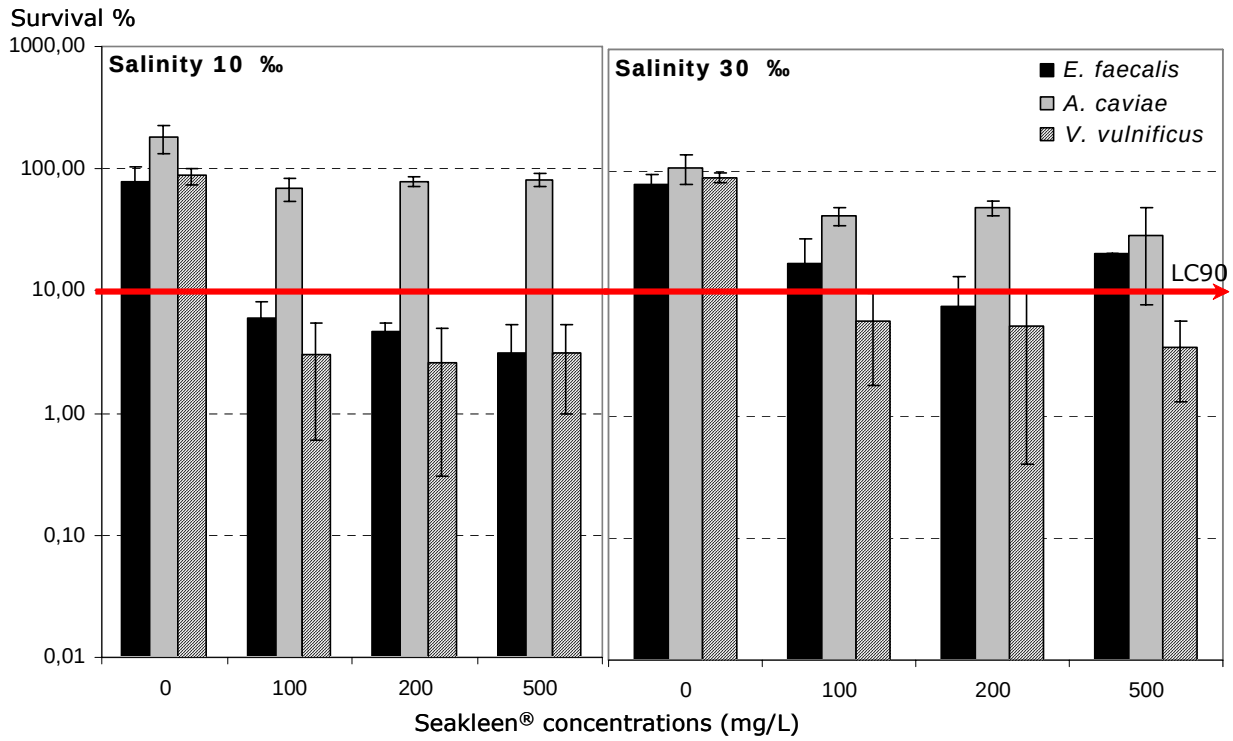


FIGURE 5.4.2 «EFFECT» OF SEAKLEEN® ON *Enterococcus faecalis, Aeromonas caviae* AND *Vibrio vulnificus*. Incubation was performed for 24 hours at 17°C in darkness in 10 PSU and 30 PSU filtered seawater. *Survival percent are mean values of two or three independent experiments and standard deviations are indicated*.

TABLE 5.4.2 LC90 OF SEAKLEEN®.

	10 PSU seawater	**30 PSU seawater**
Enterococcus faecalis	100 mg/L	100 mg/L
Aeromonas caviae	> 500 mg/L	500 mg/L
Vibrio vulnificus	100 mg/L	100 mg/L

5.4.3 Mexel® assay

Concentrations of Mexel® were estimated by a colorimetric test provided by the manufacturer according to its instruction. Each time the measured concentration in filtered seawater was lower than the one prepared. According to the manufacturer, this phenomenon was due to bounding of Mexel® to organic substances present in the water. Indeed in distilled water, no loss of measurable concentration was observed (data not shown). Moreover the loss of measurable concentration was a function of the prepared concentration: the more the solution was concentrated the more the measured concentration was below the prepared one. For example, for prepared concentrations of 2 mg/L, 5 mg/L and 10 mg/L, no loss, 40 % and 50 % of loss was observed respectively. It is important to note that the efficient active substances are not bound and thus the measured value reflects the level of active substances in the water. Concentrations mentioned below are those which have been prepared and not those that were measured.

Mexel® is effective at concentrations below 10 mg/L on all strains, except *A. caviae* in 30 PSU seawater (Figure 5.4.3 and Table 5.4.3). As for Seakleen®, *E. faecalis* and *V. vulnificus* display the same behaviour: for both salinities used the survival dramatically decreases to 1% at 2 mg/L of Mexel®. On the other hand, *A. caviae* is more resistant to Mexel® and its survival depends on the salinity of the seawater. In fact in 10 PSU seawater LC90 is obtained for concentrations ranging between 2 and 10 mg/L whereas in 30 PSU seawater 50 mg/L of Mexel® is required to yield 99 % of mortality. Other experiments (data not shown) displayed that at 20 mg/L the survival percent falls to 1% after 48h of incubation (as well as after 7 days) suggesting that at this concentration LC90 is finally reached but these incubation times are too long considering ship's activities. On the other hand incubation time has no significant impact on survival in 10 PSU seawater indicating that after 24 hours the biocide effect is completed.

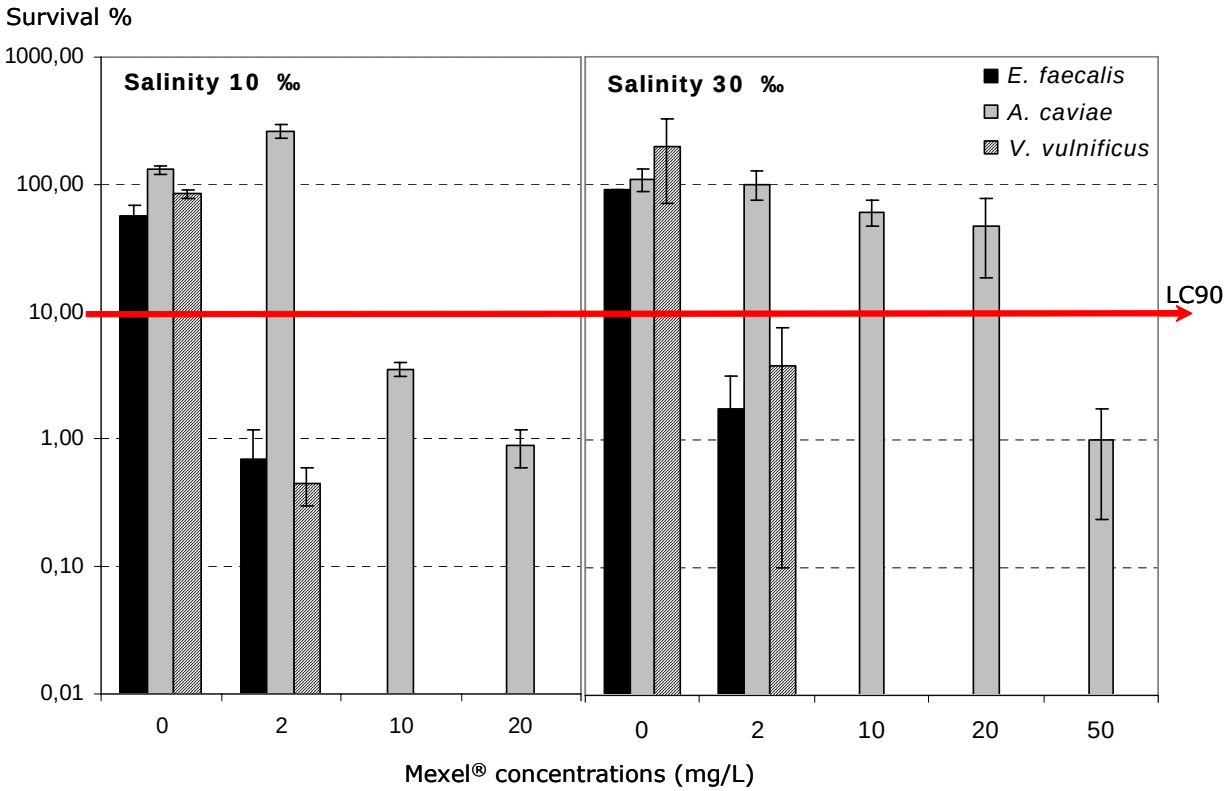


FIGURE 5.4.3 «EFFECT» OF MEXEL® ON *Enterococcus faecalis, Aeromonas caviae* AND *Vibrio vulnificus*. Incubation was performed for 24 hours at 17°C in darkness in 10 PSU and 30 PSU filtered seawater. Survival percent are mean values of two independent experiments and standard deviations are indicated.

TABLE 5.4.3 LC90 OF MEXEL®.

	10 PSU seawater	**30 PSU seawater**
Enterococcus faecalis	< 2 mg/L	< 2 mg/L
Aeromonas caviae	2 < LC90 < 10 mg/L	20 < LC90 < 50 mg/L
Vibrio vulnificus	< 2 mg/L	≤ 2 mg/L

5.4.4 Preliminary conclusions

Altogether these data indicate that PeraClean® Ocean and Mexel® seem to be the most effective biocides against bacteria. First LC90 (or more) is reached for all tested bacteria at concentrations < 50 mg/L in 24 hours whereas at least 100 mg/L is required with SeaKleen® and more than 48 hours of incubation is needed to potentially observe LC90 for *Aeromonas caviae*.

This last result is consistent with a previous study which showed that 100 mg/L to 200 mg/L of SeaKleen® are required to inhibit regrowth of *E. coli*, *S. aureus*, *L. innocua* and *V. alginolyticus* after 48h of exposure (Gregg and Hallegraeff, 2007). Moreover the results obtained with Mexel® match with the concentrations recommended by manufacturer which are 30 to 50 mg/L. Secondly PeraClean® Ocean and Mexel® are effective on the three tested bacteria which is not the case of Seakleen® since tested concentrations have no effect on *A. caviae*. Thirdly, in these laboratory tests, these two biocides (as well as SeaKleen®) do not need any specific structures to be mixed to the seawater. Fourthly the efficiency of these two substances is modified regarding the salinity of the seawater: with PeraClean® Ocean, *E. faecalis* and *V. vulnificus* are sensitive to the salinity whereas with Mexel®, only *A. caviae* is susceptible.

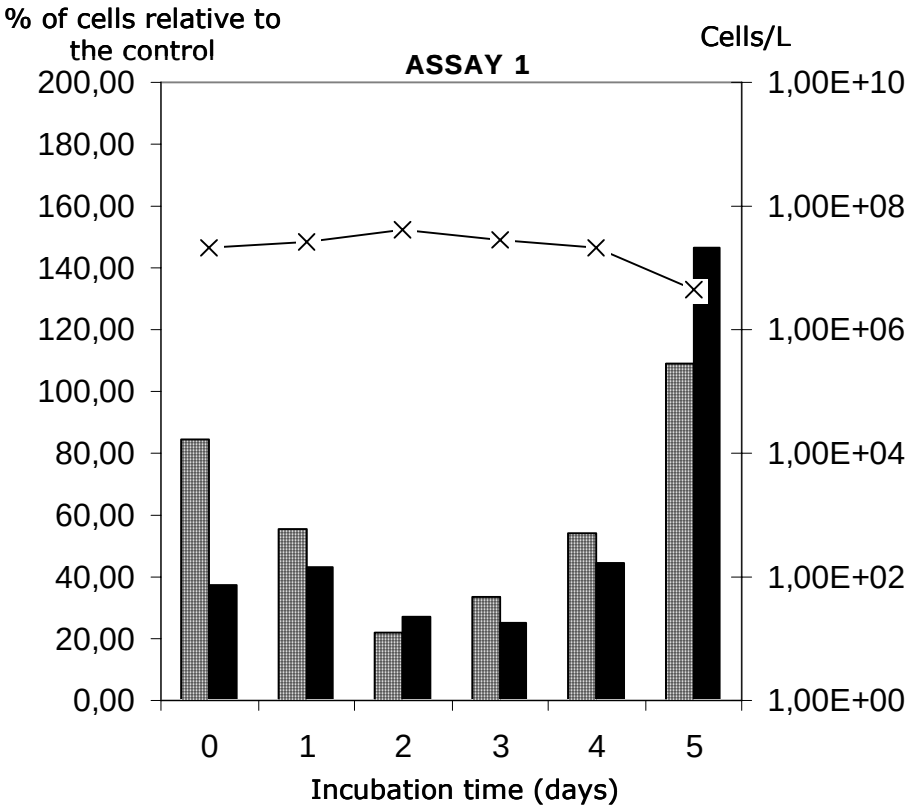
5.5 Efficiency against phytoplankton

5.5.1 PeraClean® Ocean assay

At low concentrations of PeraClean® Ocean (0,1 and 0,2 mg/L), an inhibition of growth of the treated *T. suecica* compared to the control was observed during the first two days (Figure 5.5.1-A). However the number of treated cells start to increase at day 3 reaching levels above the control at day 5 (percent of cells relative to the control > 100 %). This suggests that these concentrations are probably not sufficient to kill all the *T. suecica* cells and that the surviving cells showed regrowth when the biocide was degraded.

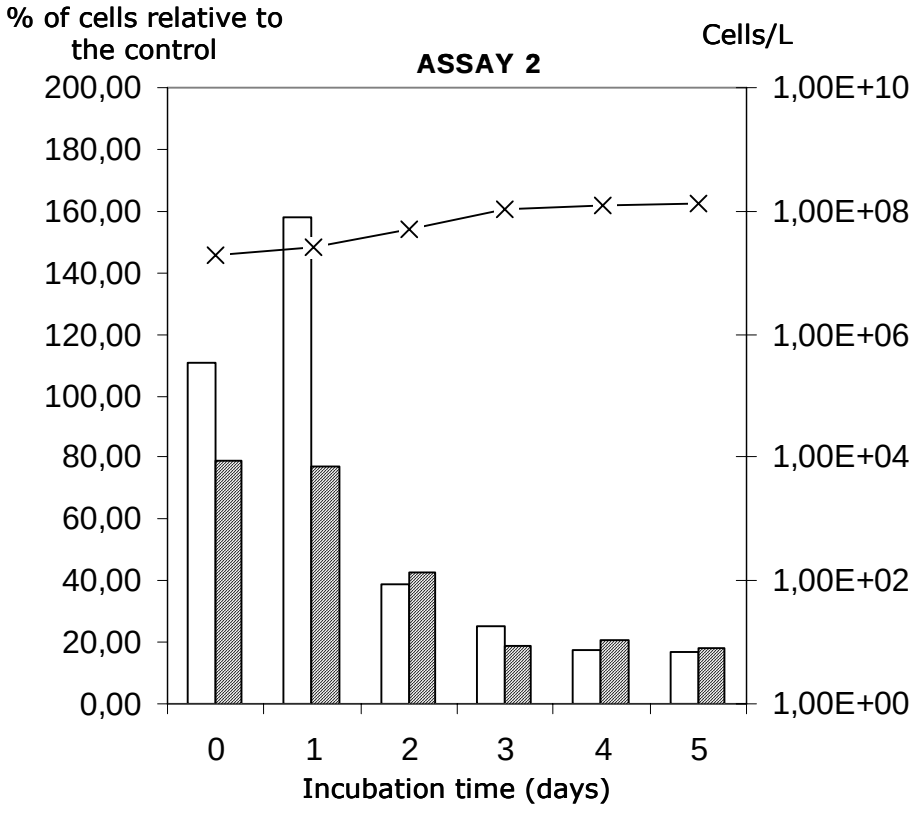
Higher concentrations (10 and 100 mg/L) induced a clear inhibition of growth in *T. suecica* compared to the control (Figure 5.5.1-A): at 10 mg/L an inhibitory effect can be seen from the second day whereas at 100 mg/L inhibition occurs from the beginning. A similar effect was also observed for *A. minutum* from day 0 (Figure 5.5.1-B) but seems to be less evident.

A/ *T. SUECICA*



PeraClean® Ocean concentrations:

- 0,1 mg/L
- 0,2 mg/L
- 10 mg/L
- 100 mg/L



B/ *A. MINUTUM*

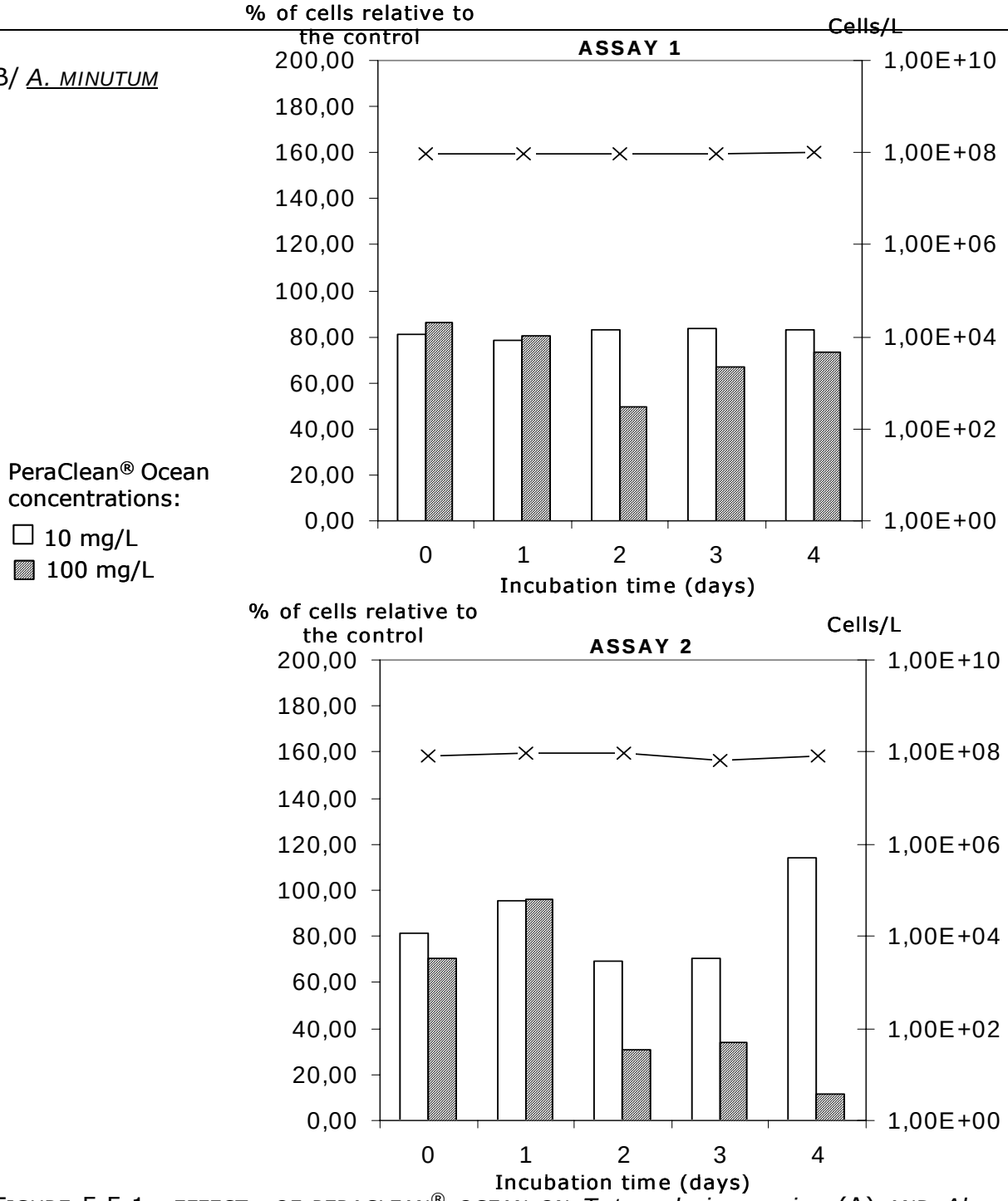


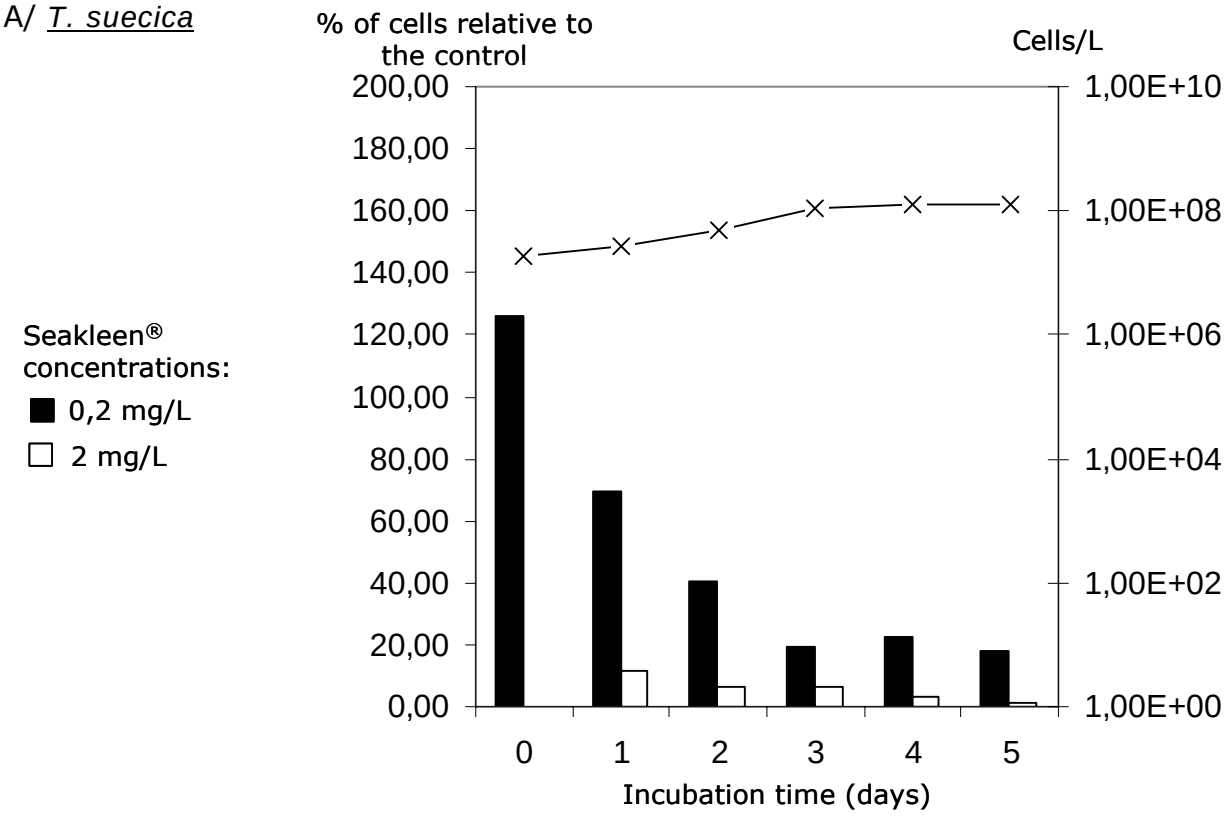
FIGURE 5.5.1 «EFFECT» OF PERACLEAN® OCEAN ON *Tetraselmis suecica* (A) AND *Alexandrium minutum* (B) GROWTH. Incubation was performed at 17°C in darkness in 30 PSU filtered seawater and growth was followed for 4 or 5 days. Each bar represents the number of cells in the treated assay as percent of that in the control (left axis) at different PERACLEAN® OCEAN concentrations (squared bars: 0,1 mg/L; black bars: 0,2 mg/L; white bars: 10 mg/L and hatched bars: 100 mg/L). The solid line shows the growth in the control over time as the absolute number of cells/L (right axis). *Each value is a mean of triplicates. Independent assays are represented.*

5.5.2 SeaKleen® assay

SeaKleen® had a strong effect on the two phytoplanktonic species at the two tested concentrations. For *T. suecica*, the recommended concentration by the manufacturer (2 mg/L) was efficient from the beginning whereas at 0,2 mg/L inhibition was observed from day 1 (Figure 5.5.2-A). No regrowth occurs suggesting that cells died in the treatment.

SeaKleen® also inhibited the growth of *A. minutum* from the beginning of the experiment at the two tested concentrations although some regrowth was observed at 0,2 mg/L from day 3 (Figure 5.5.2-B).

A/ *T. suecica*



B/ *A. minutum*

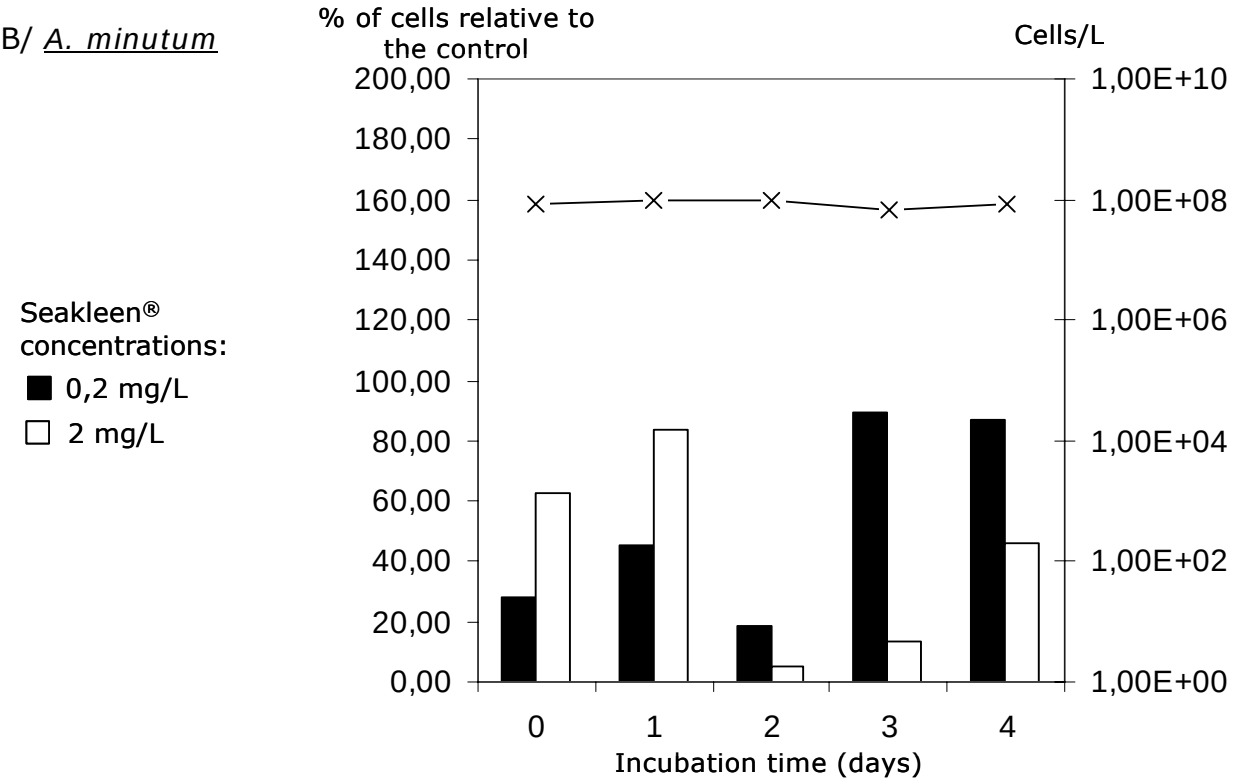
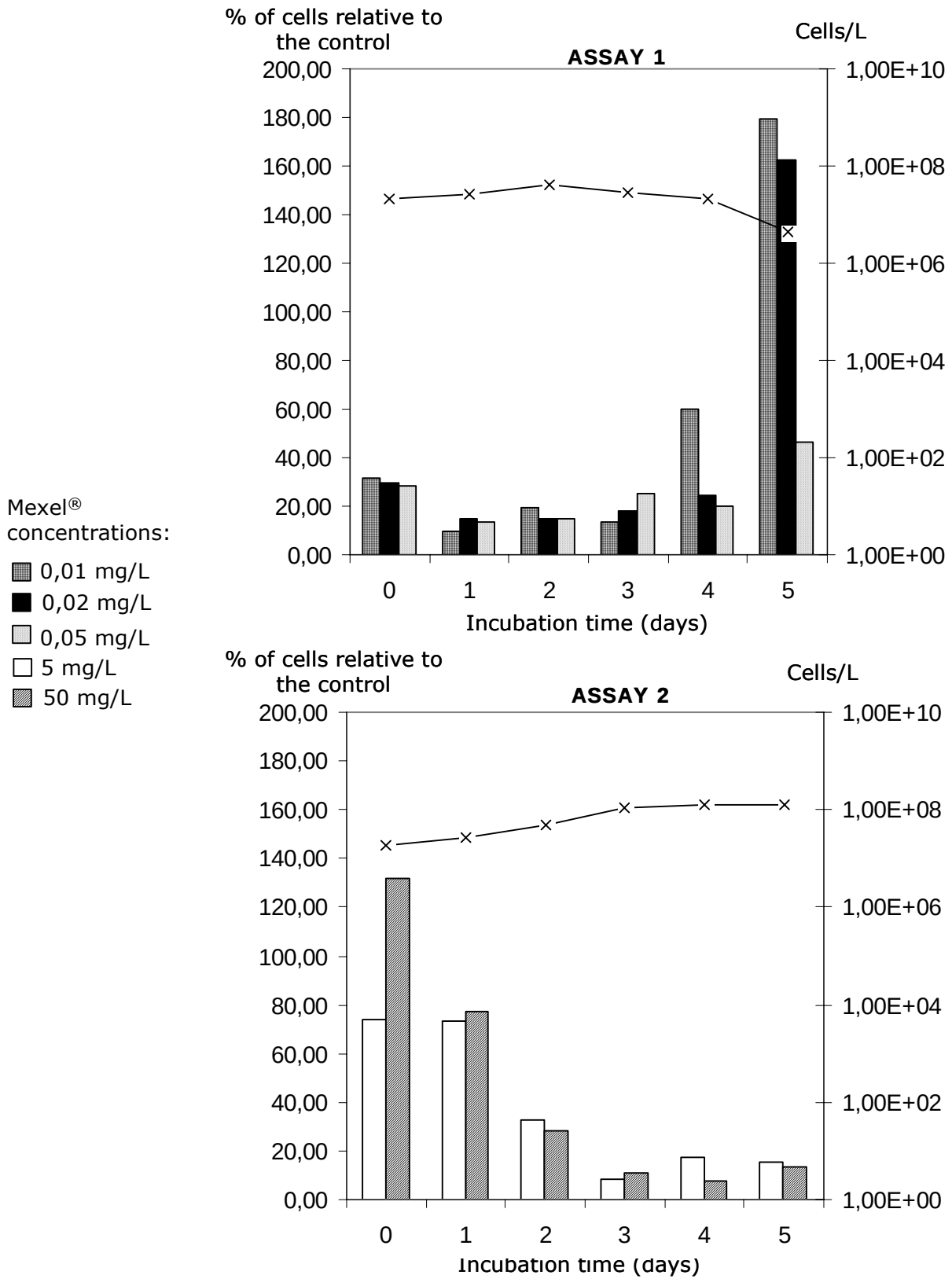


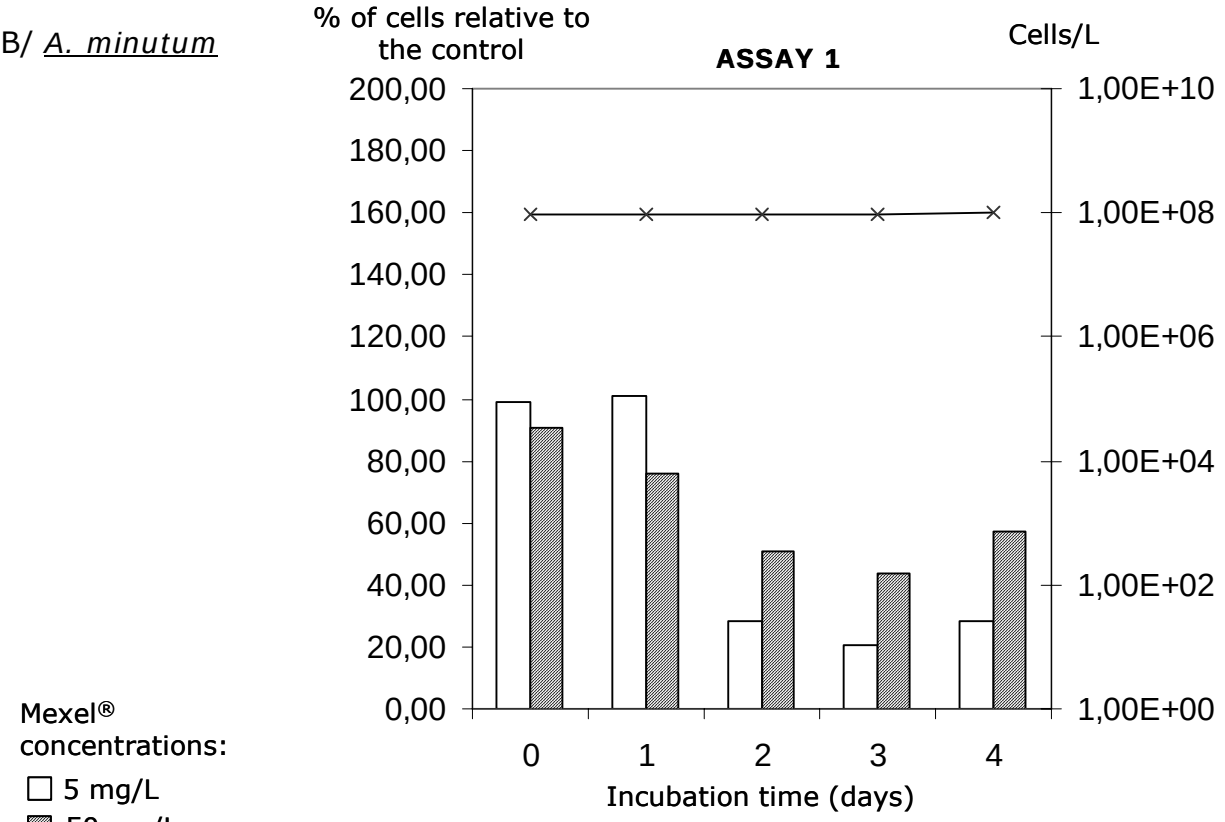
FIGURE 5.5.2 «EFFECT» OF SEAKLEEN® ON *Tetraselmis suecica* (A) AND *Alexandrium minutum* (B) GROWTH. Incubation was performed at 17°C in darkness in 30 PSU filtered seawater and growth was followed for 4 or 5 days. Each bar represents the number of cells in the treated assay as percent of that in the control (left axis) at different SEAKLEEN® concentrations (black bars: 0,2 mg/L and white bars: 2 mg/L). The solid line shows the growth in the control over time as the absolute number of cells/L (right axis). *Each value is a mean of triplicates. Independent assays are represented.*

5.5.3 Mexel® assay

Low concentrations of Mexel® (0,01; 0,02; 0,05 mg/L) efficiently inhibited the growth of *T. suecica* from day 0 (Figure 5.5.3-A). However some regrowth occurred from day 4 at 0,01 mg/L and from day 5 at the other concentrations. At 5 and 50 mg/L of Mexel® inhibition of growth was clear from day 2 and no regrowth was observed suggesting that all cells died.
For *A. minutum* 50 mg/L of Mexel® showed a strong inhibitory effect and no regrowth arose, although there was some variability between the consecutive assays (Figure 5.5.3-B). At this concentration, however cells were highly damaged and probably dead (Figure 5.5.3-C).
At 5 mg/L, results from the two assays differ greatly, being difficult to summarise: in one case there is no regrowth and in the second case some regrowth occurred.
A/ *T. suecica*

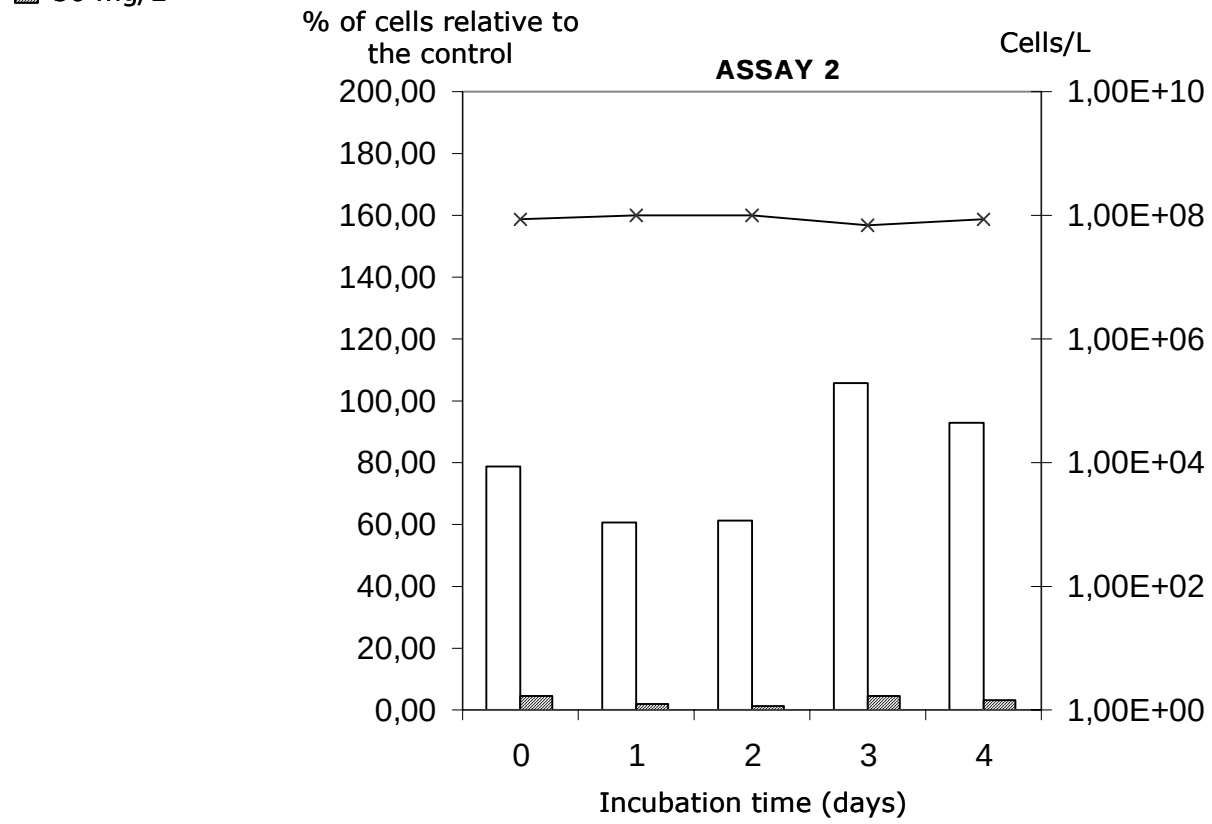

% of cells relative to the control
Cells/L
ASSAY 1
200,00
180,00
160,00
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
1,00E+00
0
1
2
3
4
5
Incubation time (days)
Mexel® concentrations:
0,01 mg/L
0,02 mg/L
0,05 mg/L
5 mg/L
50 mg/L
% of cells relative to the control
Cells/L
ASSAY 2
200,00
180,00
160,00
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
1,00E+00
0
1
2
3
4
5
Incubation time (days)

B/ *A. minutum*



Mexel® concentrations:

- ☐ 5 mg/L
- ▨ 50 mg/L



C/ *A. minutum cell*

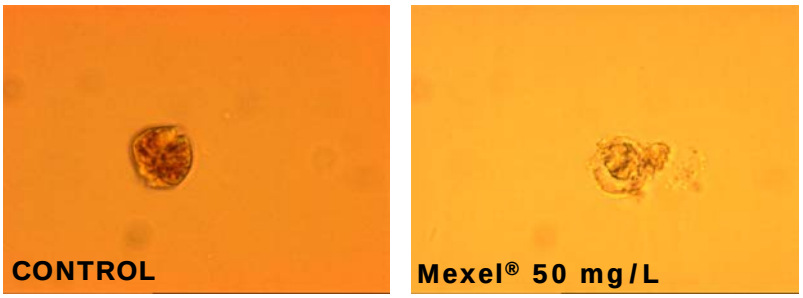


FIGURE 5.5.3 «EFFECT» OF MEXEL® ON *Tetraselmis suecica* (A) AND *Alexandrium minutum* (B) GROWTH AND CELL (C). Incubation was performed at 17°C in darkness in 30 PSU filtered seawater and growth was followed for 4 or 5 days. A, B: Each bar represents the number of cells in the treated assay as percent of that in the control (left axis) at different MEXEL® concentrations (squared bars: 0,01 mg/L; black bars: 0,02 mg/L; dotted bars: 0,05 mg/L; white bars: 5 mg/L and hatched bars: 50 mg/L). The solid line shows the growth in the control over time as the absolute number of cells/L (right axis). *Each value is a mean of triplicates. Independent assays are represented.* (C) Microscopic observation of an *A. minutum* cell *(X100).*

5.5.4 Preliminary conclusions

PeraClean® Ocean appears very efficient at the recommended 100 mg/L concentration. However it depends on the development stage of the culture (or bloom) as some re-growth is observed. One of the components is peracetic acid with a short life/efficiency when used alone. It may be necessary to use 400 mg/L to kill cysts, according to literature (Gregg and Hallegraeff, 2007).

SeaKleen® is efficient at 2 mg/L on the two tested phytoplanktonic species, and this is consistent with literature results (Gregg and Hallegraeff, 2007). Moreover, it may be necessary to use 6 or even 10 mg/L to inactivate cysts, according Gregg and Hallegraeff (2007).

Mexel® seems to have a good effect at the 50 mg/L recommended concentration against vegetative phytoplankton. However no literature data are available on eventual cysts in-activation and further studies have to be performed to check efficiency against cysts.

5.6 Efficiency against zooplankton

5.6.1 PeraClean® Ocean assay

The mortality of *Artemia* increased rapidly with increasing Peraclean® Ocean concentrations, and 96–100% mortality was achieved at the highest concentration tested (800 mg/L) (Figure 5.6.1). In the lower salinity, mortality rates were generally higher compared with those in the higher salinity (Figure 5.6.1); this effect was significant or marginally significant in all cases ($t = 4.4$, $df = 4$, $p = 0.01$; Mann-Whitney $U = 0.0$, $p = 0.04–0.05$) except in the "after-effect" test at the lowest concentration (200 mg/L). However, this effect of higher mortality in the lower salinity may be partly caused by higher initial PAA concentrations in 10 PSU than in 30 PSU (Table 5.6.1).

In line with the higher mortality rates in the lower salinity, the LC50 and LC90 concentrations in 10 PSU were lower. 24 h LC50 was determined to be 255 mg/L in 10 PSU and 327 mg/L in 30 PSU, while the LC90 values were 342 mg/L and 470 mg/L in 10 and 30 PSU, respectively.

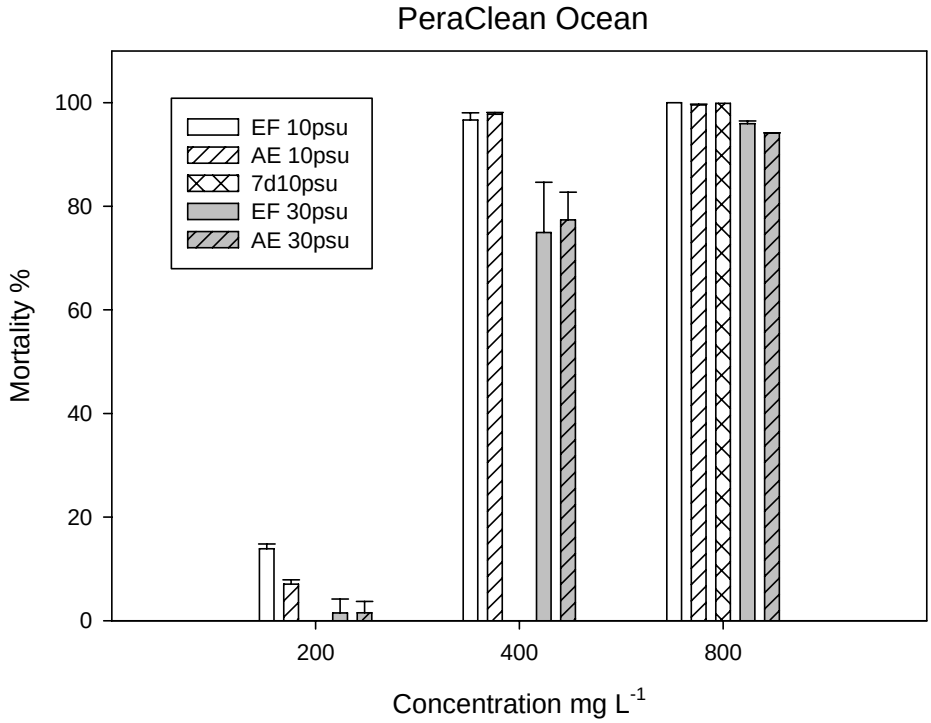


FIGURE 5.6.1 MORTALITY OF ARTEMIA IN THE BIOASSAYS WITH PERACLEAN® OCEAN. EF: MORTALITY IN THE «EFFECT» TEST, AE: MORTALITY IN THE "AFTER-EFFECT" TEST, 7D: MORTALITY AFTER A RETENTION PERIOD OF 7 DAYS.

Based on concentration measurements with the rapid measurement kit (Merckoquant peracetique test), PAA degraded rapidly in the treated water (Table 5.6.1). In the higher salinity, the initial PAA concentrations (20–100 mg/L) were reduced already within 24 h to undetectable levels (0 mg/L). In the lower salinity, on the other hand, initial concentrations were higher, and residual PAA levels of 3–10 mg/L were observed after 24 h. After 7 days no detectable levels of PAA were measured.

Table 5.6.1 Measured concentrations of PAA in the bioassays with *Artemia*.

Treatment		Measured concentration (mg/L)			
Salinity PSU	Added concentration (mg/L)	Initial	After 24 h	After 48 h	After 7 days
10	30	30–40	3	2	ND
30	30	20–25	0	ND	ND
10	60	60	6	0	ND
30	60	45	0	ND	ND
10	120	120–160	10	2	0
30	120	100	0	ND	ND

ND: Not Determined.

5.6.2 SeaKleen® assay

Mortality of *Artemia* increased with increasing SeaKleen® concentrations, and 95–97% mortality was achieved at the highest concentration tested (8 mg/L) (Figure 5.6.2). Lower mortality rates were observed in the higher salinity at 4 mg/L (Mann-Whitney U = 0.0, p = 0.046–0.05). From the mortality data, the 24 h LC50 values were determined to be 3.1 mg/L and 3.6 mg/L in 10 and 30 PSU, respectively, while the LC90 values were 5.6 mg/L (10 PSU) and 5.8 mg/L (30 PSU).

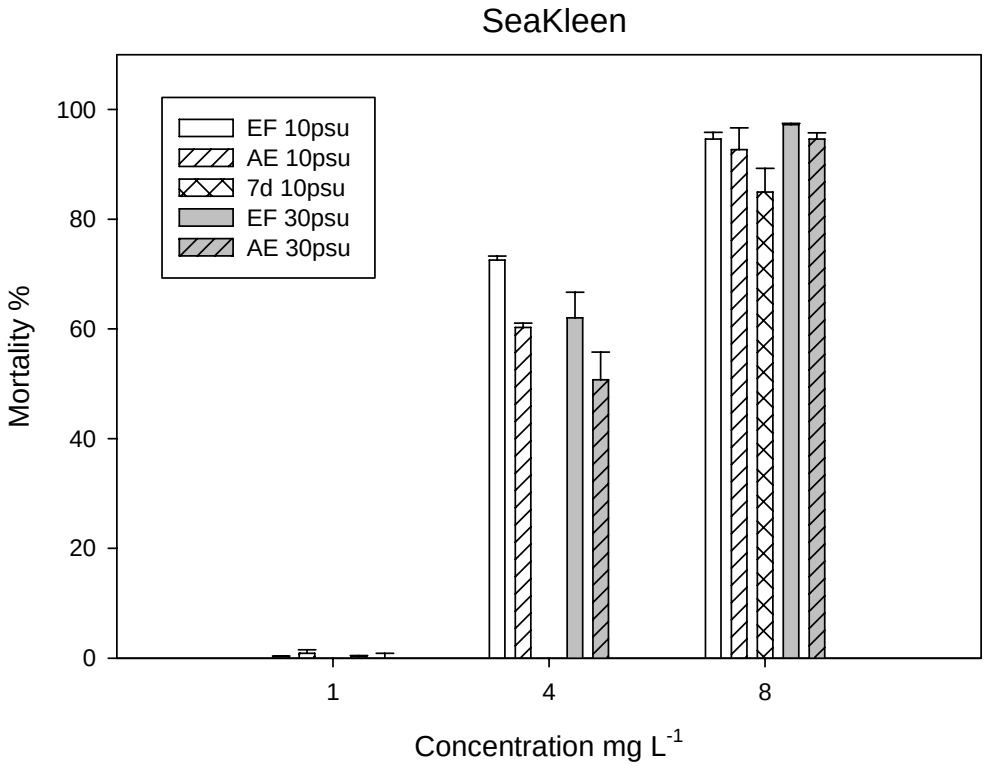


FIGURE 5.6.2 MORTALITY OF *ARTEMIA* IN THE BIASSAYS WITH SEAKLEEN®.

5.6.3 Mexel® assay

Mortality of *Artemia* increased steadily with increasing Mexel® concentrations, and 99–100% mortality was achieved at the highest concentration tested (50 mg/L) (Figure 5.6.3). In most cases, the biocide efficiency was not affected by salinity. However, significant differences between the lower and higher salinities were observed at the highest concentration in the «effect» test ($t = 7.1$, $df = 4$, $p = 0.002$) and at 5 mg/L in the "after-effect" test ($t = 6.6$, $df = 4$, $p = 0.003$). In addition, at 5 mg/L there was a marginal significance in the «effect» test (Mann-Whitney $U = 0.0$, $p = 0.05$). In all these cases, mortality was higher in the higher salinity.

A field concentration measurement kit was used to check the initial Mexel® concentrations as well as concentrations after 24 h and 7 days. The values obtained were always 3 to 5 times lower than intended, but this should be the case according to the manufacturer, since Mexel® bounds to organic substances present in the water resulting in loss of measurable concentration. However, the efficient active substances are not bound and thus the measured value should reflect the level of active substances in the water. Moderate degradation in the measured concentrations was detected within the first 24 h, but after 7 days the concentrations had not decreased further (Table 5.6.3).

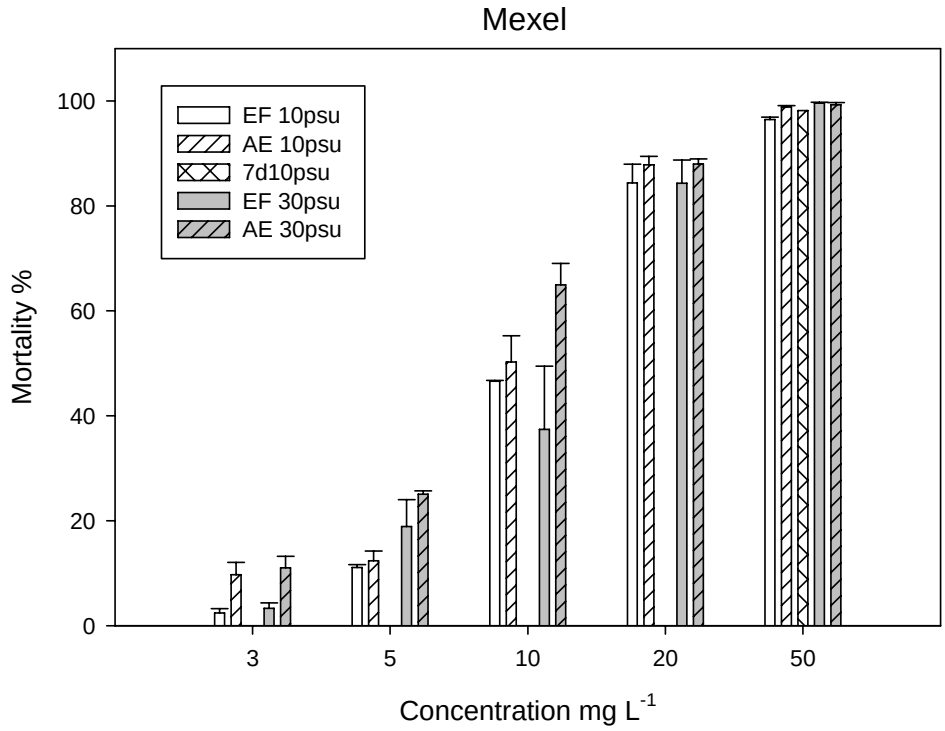


FIGURE 5.6.3 MORTALITY OF *ARTEMIA* IN THE BIOASSAYS WITH MEXEL®.

TABLE 5.6.3 MEASURED CONCENTRATIONS OF MEXEL® IN THE BIOASSAYS WITH *ARTEMIA*.

Treatment		Measured concentration (mg/L)		
Salinity PSU	Added concentration (mg/L)	Initial	After 24 h	After 7 days
30	3	1	1	ND
30	5	1	1	ND
30	10	3	2	ND
30	20	4	4	ND
10	50	10-20	4-6	4
30	50	10	6	ND

ND: Not Determined.

The total number of individuals (live and dead) decreased with increasing biocide concentration, indicating that some individuals were decomposed during the treatment. This suggests that the mortality rate (determined as the ratio of dead individuals to all individuals) was somewhat underestimated at the highest concentrations. A similar effect (i.e. loss of dead individuals) did not occur in bioassays with the other two biocides.

From the bioassay results, 24 h LC50 was determined to be 10±<1 mg/L (± 95% confidence intervals) in 10 PSU and 11±2 mg/L in 30 PSU, while the LC90 values were 23±2 mg/L and 26±10 mg/L in 10 and 30 PSU, respectively.

5.6.4 Preliminary conclusions

Of the three biocides tested, SeaKleen® was the most efficient against *Artemia* in terms of the concentration needed to achieve 90% mortality: LC90 for SeaKleen® was only 5.6–5.8 mg/L, while the values for the other biocides were 1–2 orders of magnitude higher (23–26 mg/L for Mexel® and 340–470 mg/L for PeraClean® Ocean). This is in line with previous studies, in which SeaKleen® concentrations of 0.5–1.5 mg/L have been found to be efficient in killing zooplankton (Wright and Dawson, 2001). In comparison, concentrations as high as 50–800 mg/L were needed to achieve similar mortality rates with PeraClean® Ocean (Fuchs and de Wilde, 2004). No previous data for Mexel® are available. It should be noted, however, that according to the manufacturer, the effective concentration of Mexel® is revealed by the measurement kit, and these concentrations were in all cases 3–5-fold lower than the intended concentrations.

In terms of the most efficient biocide, the results for *Artemia* differ from those obtained for bacteria. SeaKleen® proved to be effective on *Artemia* in a concentration two orders of magnitude lower than that required for bacteria, while in the case of Peraclean® Ocean, one order of lower concentration was sufficient for bacteria compared with that needed for *Artemia*.

In previous studies with SeaKleen®, a powder-like form was used, whereas we used a liquid form. 100% mortality in aquatic invertebrates, namely the copepod *Eurytemora affinis*, the zebra mussel *Dreissena polymorpha*, the amphipod *Leptocheirus plumulous* and the mussel *Mytilus galloprovincialis*, was achieved with the powder-like SeaKleen® at 0.5–2.0 mg/L (Wright and Dawson 2001, Cutler *et al*., 2004). In addition, a slightly different formulation (menadione nicotinamide bisulphite) yielded 100% mortality within 48 h at 1 mg/L in *M. galloprovincialis* and at 5 mg/L in *Artemia salina* (Faimali *et al*., 2006). This is close to the 24 h LC90 (5.6–5.8 mg/L) obtained in our study with the liquid form of SeaKleen®, indicating that the efficiency was not dramatically affected by the different formulation of the substance.

In previous bioassays with Peraclean® Ocean, concentrations of 50–200 mg/L served to produce 100% mortality in 24 h in a variety of zooplankton taxa, and 200–400 mg/L were sufficient to reach 100% mortality in zooplankton in about 1–2 h. For *Artemia* nauplii, 400 mg/L yielded 100% mortality in 11–36 h, depending on water salinity and temperature (Fuchs and de Wilde, 2004), being in the range of the LC90 values in this study. Doubling the dosage to 800 mg/L resulted in a similar (100%) mortality in 4–19 h (Fuchs and de Wilde, 2004). In those bioassays, the highest efficiency was achieved in the lowest salinity and warmest temperature used (13.5 PSU, 32°C), while the lowest efficiency was recorded in the highest salinity and lowest temperature (31 PSU, 24°C). Our results are in accordance with this observation of lower efficiency in higher salinity.

This salinity effect was consistent throughout the bioassays, i.e., in the lower salinity, also the degradation of PAA was slower as well as the reduction in toxicity with time, in comparison with measurements in the higher salinity. The effect of salinity was not as clear with the other two biocides.

A crucial issue in the use of biocides in ballast water treatment systems is the notably lower efficacy of the tested biocides against chemically more resistant inactive resting stages of aquatic species, and efficacy against living cells within ballast tank sediments is a matter of even a greater concern. For example, to inactivate resting stages (eggs) of zooplankton (rotifers, copepods, cladocerans), notably higher concentrations (24 h LC90 2.6–8.7 mg/L) of SeaKleen® were constantly needed, compared with those yielding a 90% mortality in active life stages (Raikow *et al*., 2006). For *Artemia* resting eggs, LC90 was achieved at 6.6–11.9 mg/L SeaKleen® (Raikow *et al*., 2006). Similar observations of lower biocide efficiency against inactive (cysts) vs. active life stages have been reported for phytoplankton (Gregg and Hallegraeff, 2007). Finally, SeaKleen® concentrations up to 20-fold higher (180 mg/L) than those needed to kill active life stages were necessary to inactivate cladoceran resting eggs buried in sediment (Raikow *et al*., 2006).

6 ENVIRONMENTAL ACCEPTABILITY

6.1 Environmental acceptability of peracetic acid

6.1.1 "After-effect" on bacteria

With PeraClean® Ocean, no "after-effect" is observed: whatever the concentration and the salinity all strains display a decrease of mortality or even growth (Figure 6.1.1). This data indicates that the biocide is significantly degraded in 24 hours which is confirmed by concentration measurements (data not shown) and that the remaining concentration is acceptable and even safe for the three bacteria tested.

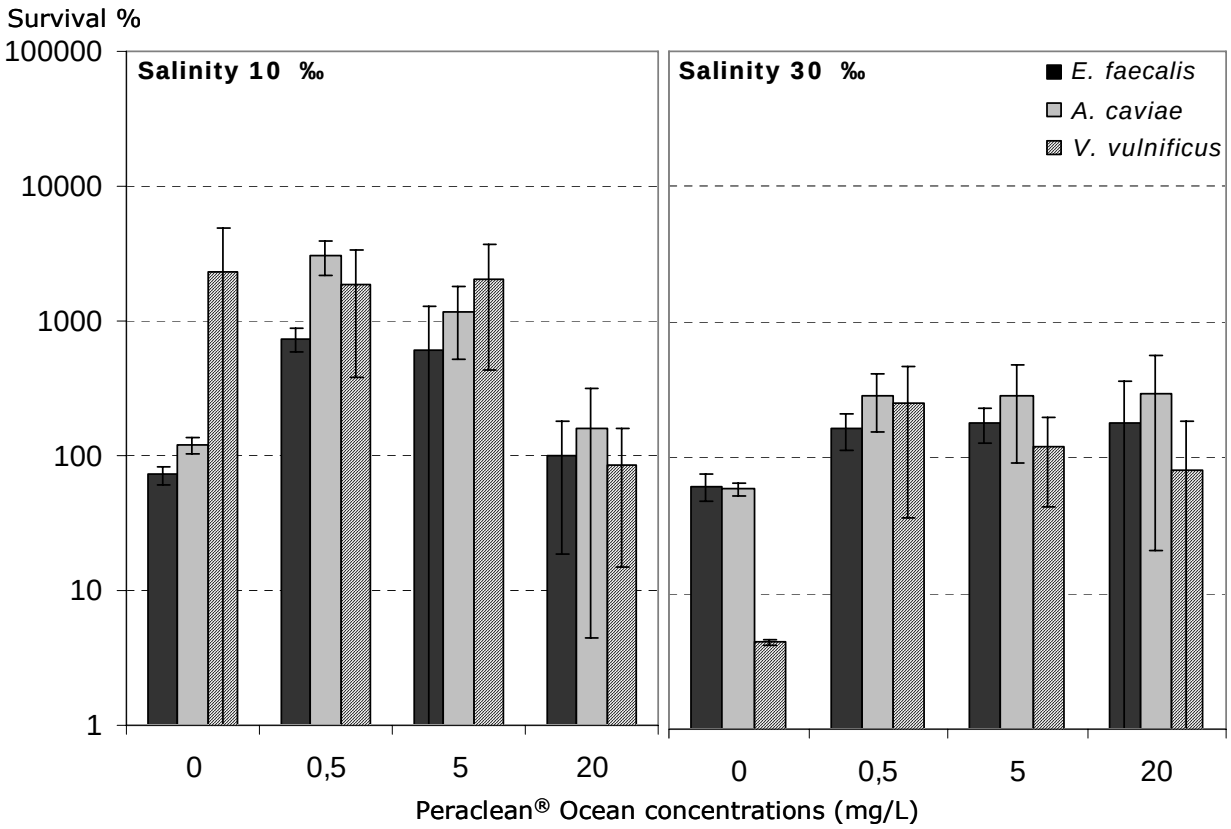


FIGURE 6.1.1 "AFTER-EFFECT" WITH PERACLEAN® OCEAN ON *Enterococcus faecalis*, *Aeromonas caviae* AND *Vibrio vulnificus*. Incubation was performed for 24 hours at 17°C in darkness in 10 PSU and 30 PSU filtered seawater. *Survival percent are mean values of three independent experiments and standard deviations are indicated.*

6.1.2 "After-effect" on zooplankton

In the "after-effect" tests with *Artemia*, in the lower salinity, mortality decreased significantly relative to the «effect» test, both at the lowest (200 mg/L; t = 5.4, df = 4, p = 0.006) and highest concentrations of Peraclean Ocean (800 mg/L; Mann-Whitney U = 0.0, p = 0.03). In addition, in 30 PSU, a marginally significant decrease was observed at the highest concentration (Mann-Whitney U = 0.0, p = 0.05). Despite statistically significant differences, the reduction in mortality was very modest at the highest concentration, from 100% to 99.5% (10 PSU) and from 95.9% to 94.1% (30 PSU) (Fig. 5-6-1), corresponding to 0.5% and 2%, respectively (Table 6.1.2). In contrast, at the lowest concentration in 10 PSU, the reduction was as high as 49% (Table 6.1.2). Degradation and residual toxicity of the highest concentration in 10 PSU was also monitored after 7 days, but no reduction in mortality was observed (Fig. 5.6.1, Table 6.1.2). Instead, a 99.9% kill was recorded.

TABLE 6.1.2 REDUCTION (%) IN MORTALITY WITH TIME (MEAN) IN THE TESTS WITH PERACLEAN® OCEAN, RELATIVE TO MORTALITY IN THE «EFFECT» TEST. FOR EMPTY CELLS, MORTALITY WAS NOT DETERMINED.

Experiment details		Reduction in mortality %	
Salinity (PSU)	Concentration (mg/L)	"After-effect"	After 7 days
10	200 400 800	49 0 0.5	 0.1
30	200 400 800	0 0 2	

6.1.3 Ecotoxicological tests

Nine different concentrations of PeraClean® Ocean from 200 mg/L (recommended concentration for elimination of phytoplankton cysts) to 0,001 mg/L were tested in six independent assays to determine the NOEC (Non Observable Effect Concentration). Each assay corresponds to larvae obtained from the fertilization of one oyster broodstock couple.

For concentrations ranging from 200 to 0,1 mg/L, 100 % of abnormal larvae were observed compared to less than 10 % for control samples (Figure 6.1.3.1). At 0,001 mg/L, abnormalities finally decrease from 100 % to 40%. The results suggest that PeraClean® Ocean has to be diluted to a lower concentration before being released in the environment.

The sensitivity of the different individuals to pollutants is not always the same depending on the broodstocks. This parameter is controlled by exposing oyster larvae to copper sulphate ($CuSO_4$) which is the reference toxic specifically used in oyster larvae tests: the more the individuals are sensitive to pollutants the more the abnormalities percent is high following $CuSO_4$ treatment. This test was performed in parallel of biocide assays in two independent experiments (Figure 6.1.3.2). Thus, the effect of PeraClean® Ocean at 10 and 20 mg/L is the same on sensitive (100 % of abnormalities with CuSO4) and less sensitive individuals (50% of abnormalities with $CuSO_4$).

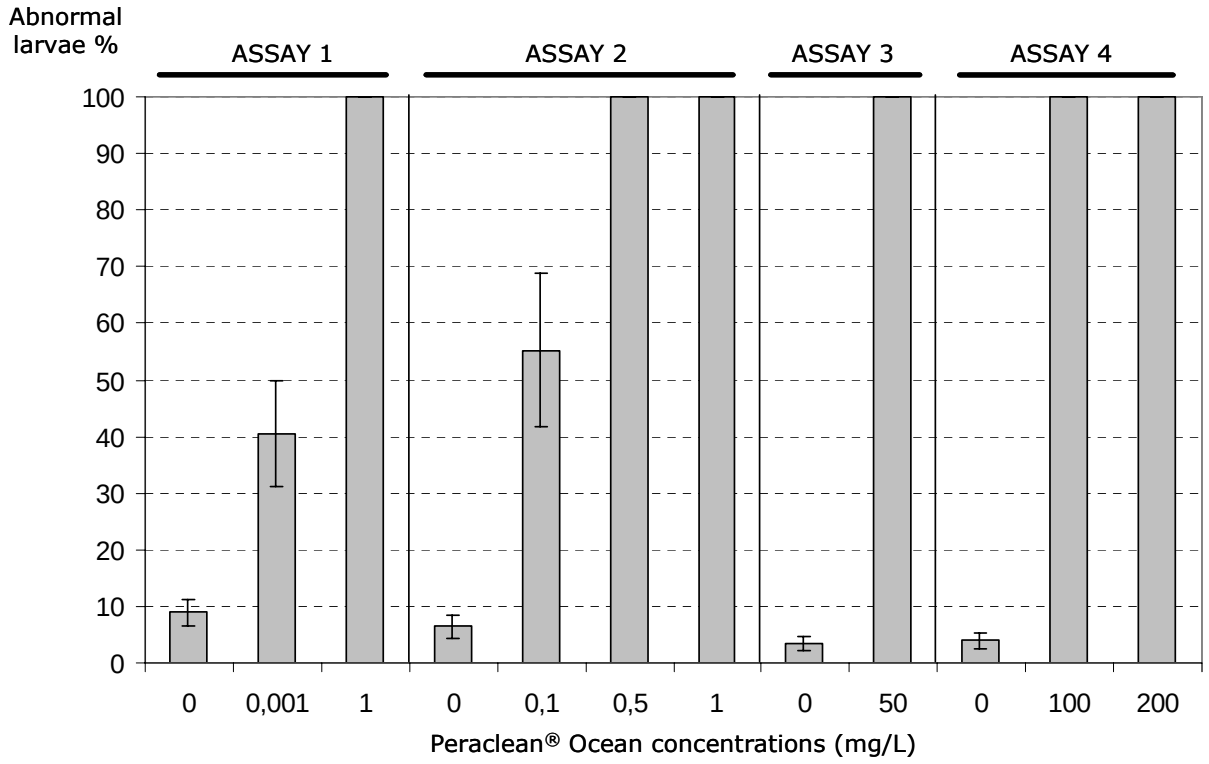


FIGURE 6.1.3.1 ECOTOXICOLOGICAL EFFECTS OF PERACLEAN® OCEAN AFTER 24 HOURS OF INCUBATION. Oyster larvae were exposed to various biocide concentrations in four independent assays and abnormal larvae at the "D-stage" were counted. *Abnormal larvae percent are mean values of five independent experiments and standard deviations are indicated. No error bars signifies five identical results.*

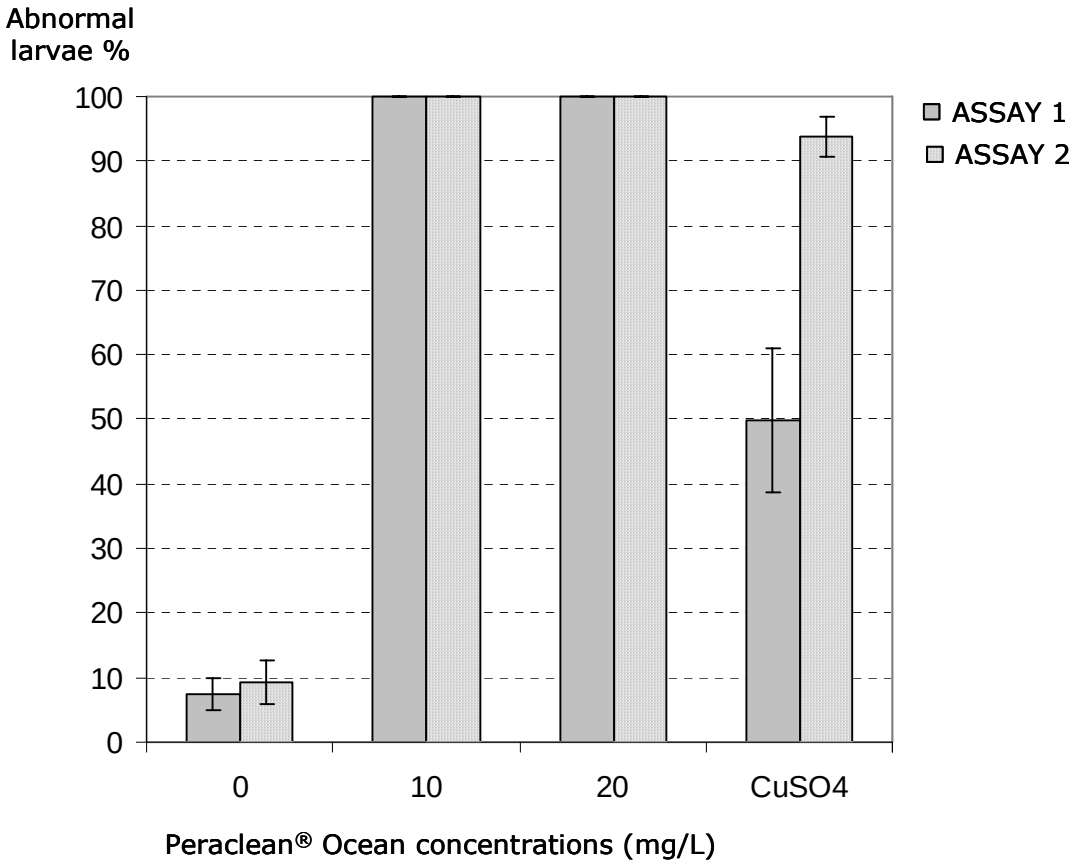


FIGURE 6.1.3.2 ECOTOXICOLOGICAL EFFECTS OF PERACLEAN® OCEAN AFTER 24 HOURS OF INCUBATION RELATIVE TO SENSITIVITY OF INDIVIDUALS. Two independent assays are represented (grey and dotted bars). Oyster larvae were exposed to 10 and 20 mg/L of biocide and to 25 µg/L of $CuSO_4$; abnormal larvae at the "D-stage" were then counted. *Abnormal larvae percent are mean values of five independent experiments and standard deviations are indicated. No error bars signifies five identical results.*

6.1.4 Preliminary conclusions on environmental acceptability of peracetic acid based biocide

The "after-effect" experiments on bacteria showed that at the tested concentration, PeraClean® Ocean degrades to a level less toxic or even non-toxic to bacteria within 24 hours in dark conditions at 17°C.
In the bioassays with *Artemia* concentrations of PAA rapidly decreased to undetectable levels in 24 hours, but contrarily to bacteria, a corresponding decrease in mortality was not observed, indicating that some substances other than PAA caused high mortality in *Artemia* even after 7 days (at 800 mg/L). PeraClean® Ocean (150 mg/L) was tested as part of the SEDNA®-system in a type approval process according to the IMO guidelines (Veldhuis and Fuhr, 2008).
In those tests, peracetic acid degraded to undetectable levels (<0.5 mg/L) within ≤20 h, while degradation of hydrogen peroxide, another component of the biocide, took 54–114

hrs (Veldhuis and Fuhr, 2008). Remains of H_2O_2 most probably explain the high mortality of *Artemia* in the "after-effect" test.

It is important to note that PeraClean® Ocean concentration scales tested against bacteria and *Artemia* are totally different: 0,5 to 20 mg/L and 200 to 800 mg/L respectively. This may explain differences observed in the degradation rate. Indeed, a study by Gregg and Hallegraeff (2007) showed that Peraclean® Ocean at 200 mg/L lost toxicity against phytoplanktonic species within 3–6 weeks, while at 1000 mg/L at least 8 weeks were required. Moreover an effect linked to the organism itself has to be considered.

Finally oyster larvae tests indicate that PeraClean® Ocean should be diluted at least to 0,001 mg/L to have a minimum impact.

In the type approval tests for the SEDNA®-system, bacterial regrowth started 7–9 days after the treatment, while no tests were run to verify residual toxicity of the treated water against phytoplankton or zooplankton (Veldhuis and Fuhr, 2008). Considering the present results, there are some concerns related to the degradability and residual toxicity of this biocide, especially at high concentrations (400–800 mg/L), but the results cannot be directly compared with the SEDNA®-system type approval tests. Firstly, the SEDNA®-system uses PeraClean® Ocean at a lower concentration of 150 mg/L, being lower than the lowest concentration tested against *Artemia* here (200 mg/L, which caused only 2–7% mortality in the "after-effect" tests). Secondly, the biocide may perform differently on large (300 m^3) and small scales (10 L), and in the presence of a natural assemblage of marine organisms. Nevertheless, the recommended 24-h retention period for this substance appears not long enough to eliminate the toxic effects on all organism groups.

6.2 Environmental acceptability of vitamin K3 base

6.2.1 "After-effect" on bacteria

Whatever the salinity, survival percentage of *E. faecalis* in the "after-effect" experiment are similar to those in the "effect" one (Figure 6.2.1). This means that in 24 hours SeaKleen® is not sufficiently degraded for this species.

However decrease of mortality (32%) was observed for *V. vulnificus*, except in 10 PSU seawater with 500 mg/L of SeaKleen®. On the other hand, growth of *A. caviae* is observed at 500 mg/L treated water (10 PSU) whereas mortality is unchanged at lower concentrations. Moreover there is a decrease of mortality (30%) in 30 PSU seawater. Therefore regarding the mortality rate some degradation of the biocide must occur.
Nevertheless the estimation of the concentration of menadione (i.e. SeaKleen®) in the assay by a spectrophotometric method at 337 nm showed that the biocide was not degraded in 24 hours (data not shown).

Furthermore, the reason for growth in the presence of high concentration of Seakleen® in 10 PSU seawater remains unclear.

Interestingly growth of *A. caviae* in the control (i.e. without biocide) in the "after-effect" experiment (only) is more important in 10 PSU than in 30 PSU suggesting that the lower salinity is less restrictive for *A. caviae* development.

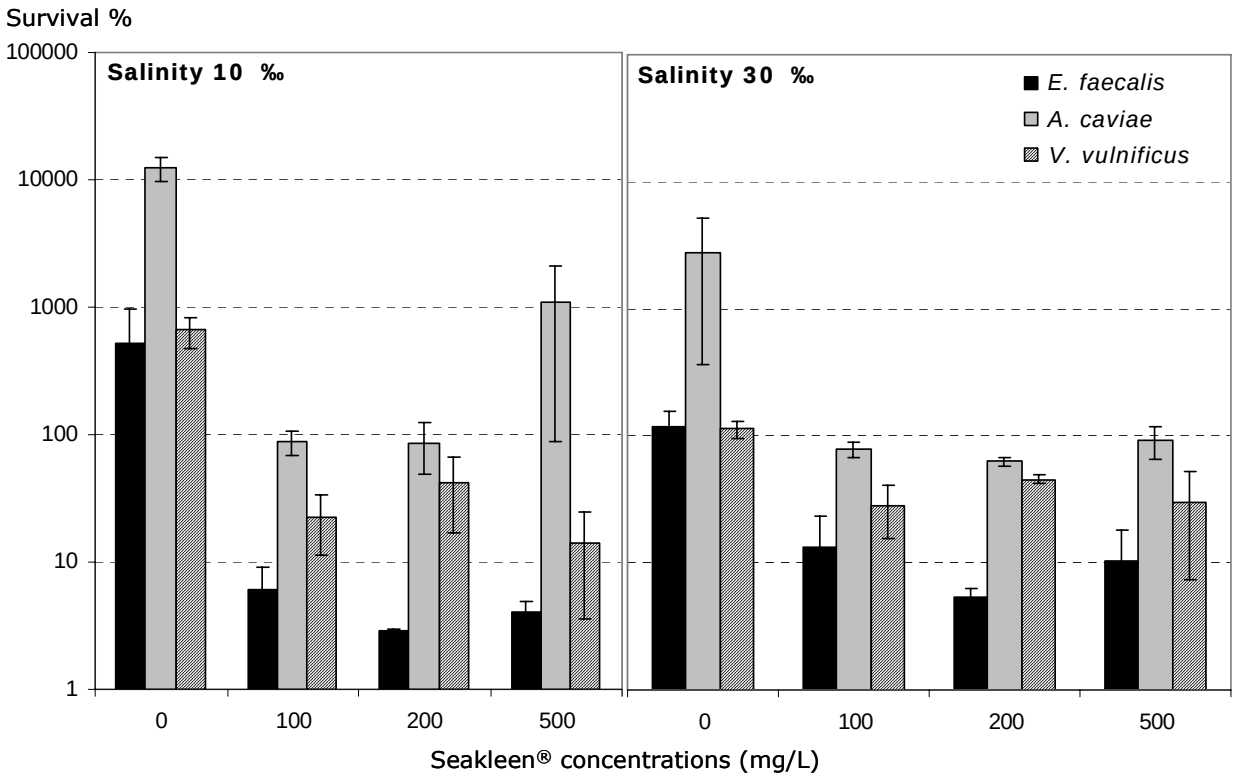


FIGURE 6.2.1 "AFTER-EFFECT" WITH SEAKLEEN® ON *Enterococcus faecalis*, *Aeromonas caviae* AND *Vibrio vulnificus*. Incubation was performed for 24 hours at 17°C in darkness in 10 PSU and 30 PSU filtered seawater. *Survival percent are mean values of three independent experiments and standard deviations are indicated.*

6.2.2 "After-effect" on zooplankton

In the "after-effect" tests with *Artemia*, mortality decreased significantly relative to the «effect» tests, from 72.6% to 60.3%, at 4 mg/L in 10 PSU ($t = 11.4$, $df = 4$, $p < 0.001$). No significant differences were observed at other concentrations, despite the high reduction percentages at 1 and 4 mg/L in the 30 PSU tests (Table 6.3.3). At the highest concentration (8 mg/L), the decrease in mortality was from 94.6% to 92.7% (10 PSU) and from 97.2% to 94.6% (30 PSU), corresponding to 2% and 3%, respectively (Table 6.2.2).

The degradation and residual toxicity of the highest concentration was monitored in both salinities after 7 days. In 10 PSU, a slight decrease in mortality was observed from 92.7% in the "after-effect" test to 84.9% in the test run 7 days later, while in 30 PSU practically no further decrease in mortality was observed. In addition, residual toxicity of the second highest concentration (4 mg/L) in the higher salinity was monitored after 14 and 28 days. During the first 14 days, mortality slightly increased from 50.7% in the "after-effect" test to 62.3% in the test run 15 days later. The last bioassay was run after one month, and at this point mortality had decreased down to 45.2%.

TABLE 6.2.2 REDUCTION IN MORTALITY WITH TIME (MEAN) IN THE TESTS WITH SEAKLEEN®, RELATIVE TO MORTALITY IN THE «EFFECT» TEST. FOR EMPTY CELLS, MORTALITY WAS NOT DETERMINED.

Experiment details		Reduction in mortality %			
Salinity (PSU)	Concen-tration (mg/L)	"After-effect"	After 7 days	After 14 days	After 28 days
10	1 4 8	0 17 2	 10		
30	1 4 8	78 18 3	 11 3	0	27

6.2.3 Ecotoxicological tests

SeaKleen® was tested at concentrations ranging from 0,2 to 0,005 mg/L (Figure 6.2.3). Each tested concentration seems to have an impact on oyster larvae development suggesting that inactivation or degradation treatments must be applied before release in the environment. Gregg and Hallegraeff (2007) also proposed to use this active substance as an emergency treatment only for exceptional situations.

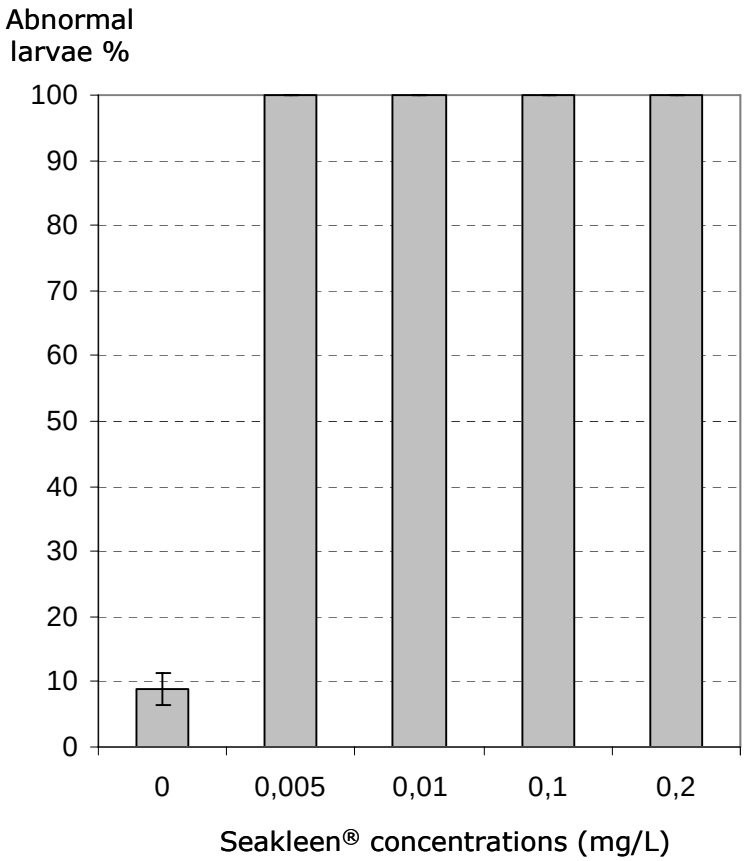


FIGURE 6.2.3 ECOTOXICOLOGICAL EFFECTS OF SEAKLEEN® AFTER 24 HOURS OF INCUBATION. Oyster larvae were exposed to various biocide concentrations and abnormal larvae at the "D-stage" were count. *Abnormal larvae percent are mean values of five independent experiments and standard deviations are indicated. No error bars signifies five identical results.*

6.2.4 Preliminary conclusions on environmental acceptability of vitamin K3 based biocide

The "after-effect" of SeaKleen® on bacteria after 24 hours of incubation varied regarding the strain, the salinity and the concentration. This means that degradation of the biocide and loss of its toxicity depends on the condition suggesting that effects of this substance would not be correctly controlled.

Regarding *Artemia*, SeaKleen® showed a slight level of reduction in efficiency after 24 hours in the experimental conditions and this was accentuated by a longer incubation (until 28 days). Interestingly, a previous study showed that SeaKleen® almost totally failed to lose toxicity during the 14-weeks monitoring period (Gregg and Hallegraeff, 2007). It is possible that modification in the form of SeaKleen® (from powder-like to liquid) has improved its degradation properties.

However, as in the case with PeraClean® Ocean, the concentrations needed to inactivate bacteria and zooplankton differed drastically (two orders of magnitude), which probably explains the differences observed in the residual toxicity against these two organism groups. Despite the reduction in toxicity demonstrated with *Artemia* at relatively low concentrations, oyster larvae tests indicate that additional treatments must be applied to reach a non-toxic level.

6.3 Environmental acceptability of alkylamine base

6.3.1 "After-effect" on bacteria

For *E. faecalis* a decrease of mortality was observed with 2 mg/L of Mexel® whatever the tested salinity was (100 % in 10 PSU and 45 % in 30 PSU seawater). Above this concentration the reduction of mortality was really slight (until 15 %) and at 50 mg/L no reduction was observed (Figure 6.3.1).

For *V. vulnificus* with 2 mg/L of Mexel® reduction was different according to the salinity: 8 % in 10 PSU and 54 % in 30 PSU (Figure 6.3.1). At higher concentrations, no significant difference was recorded between the two tested salinities: decrease in mortality was on average 14 % with 10 mg/L, 5 % with 20 mg/L and 0 % with 50 mg/L. These results suggest that for these two strains there is some degradation of the biocide after 24h but it seems to be not sufficient to reach non-toxic levels when the initial concentration is high.

In the case of *A. caviae*, no "after-effect" was detected. Although 50 mg/L of Mexel® yielded to 99,5 % of mortality (average of the two salinities) after 24 hours («effect» assay, Figure 5.4.3), the remaining concentration at this time was not lethal for *A. caviae* since no mortality was observed in the "after-effect" experiment. This last data also indicates that Mexel® is degraded in 24 hours and confirms that *A. caviae* is the most resistant bacterium to this biocide.

No decrease in the measured concentration of Mexel® was observed over time even after 6 days of incubation (Table 6.3.1). However, according to the manufacturer these concentrations did not reflect the remaining active substances but rather the degradation products of Mexel® which are also measured by the colorimetric test. These by-products are usually not efficient. This is verified on *A. caviae* but for *E. faecalis* and *V. vulnificus* the reason of the still high level of mortality remains unclear.

As it has been noted in the Seakleen® assay, growth in the control as well as in the biocide tests is stronger in 10 PSU than in 30 PSU. However in the "effect" experiment *A. caviae* displays more resistance in 30 PSU seawater (see Figure and Table 5.4.3).

In conclusion 10 PSU seawater should be more appropriate for growth in the "after-effect" experiment. On the other hand, the resistance of the bacterium in 30 PSU seawater in the «effect» experiment could be linked to a potential protector effect of NaCl at this concentration.

TABLE 6.3.1 MEASURED CONCENTRATIONS OF MEXEL® IN THE BIOASSAYS WITH BACTERIA. The two indicated values reflect the variation of the measured concentration observed within the studied strains. ND: NOT DETERMINED.

Treatment		Measured concentration (mg/L)			
Salinity PSU	Expected concentration (mg/L)	Initial	After 24 h	After 48 h	After 6 days
10	2	1-2	1	ND	ND
10	10	3-5	3-4	ND	ND
10	20	6-8	ND	6-7	6
30	2	1-2	0-1	ND	ND
30	10	3-5	ND	ND	ND
30	20	5-8	5-6	6-7	5-6

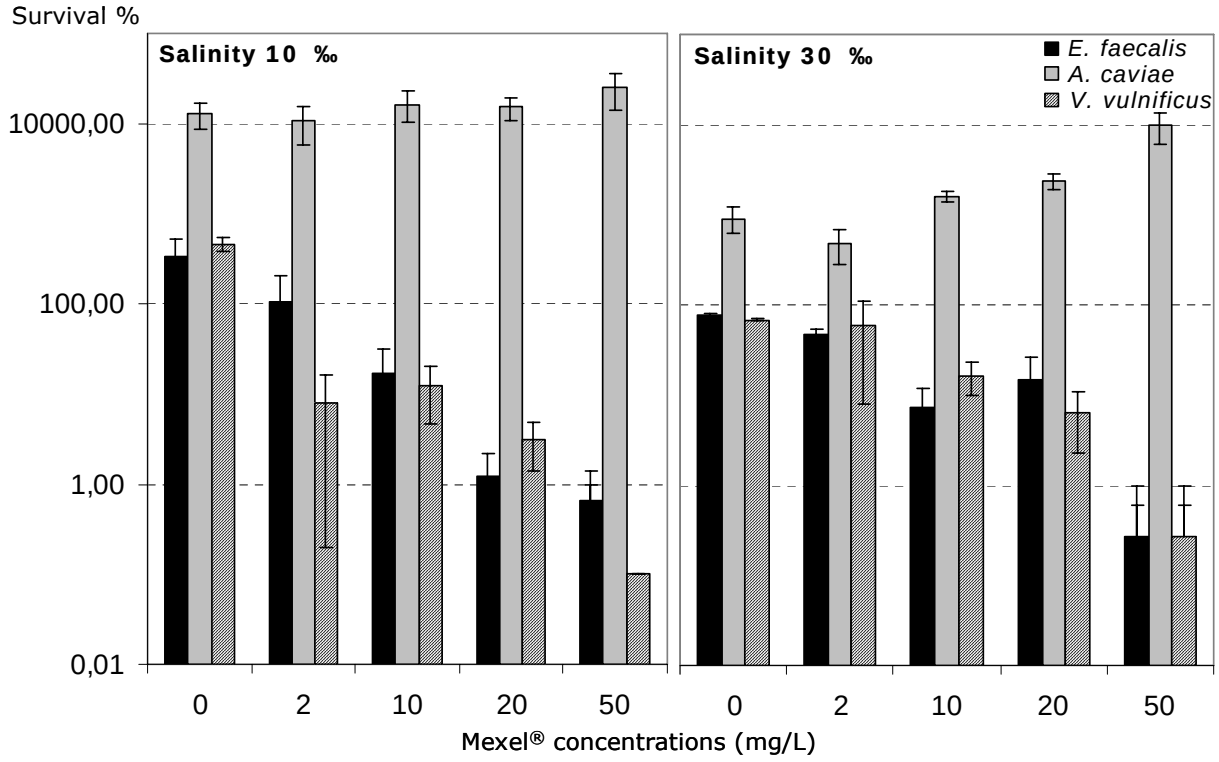


FIGURE 6.3.1 "AFTER-EFFECT" WITH MEXEL® ON *Enterococcus faecalis*, *Aeromonas caviae* AND *Vibrio vulnificus*. Incubation was performed for 24 hours at 17°C in darkness in 10 PSU and 30 PSU filtered seawater. *Survival percent are mean values of three independent experiments and standard deviations are indicated.*

6.3.2 "After-effect" on zooplankton

In the "after-effect" tests with *Artemia*, the biocide did not appear to degrade, and mortality even increased in both salinities compared with those in the «effect» tests. This difference in mortality between the «effect» and "after-effect" tests was significant at the lowest (3 mg/L; t = 3.0–3.2, df = 4, p = 0.04–0.03) and highest concentrations (10 PSU, 50 mg/L; U = 0.0 0.p = 046). Mortality rates at the highest concentration increased from 96.5% to 98.8% in 10 PSU and dropped from 99.6% to 99.3% in 30 PSU. In addition, degradation and residual toxicity of the highest concentration in 10 PSU was monitored after 7 days, but mortality had not decreased during this longer retention period (Figure 5.6.3, Table 6.3.2). Instead, a total 100% kill was recorded, indicating poor degradation properties of the biocide.

TABLE 6.3.2 REDUCTION IN MORTALITY WITH TIME (MEAN) IN THE TESTS WITH MEXEL®, RELATIVE TO MORTALITY IN THE «EFFECT» TEST. FOR EMPTY CELLS, MORTALITY WAS NOT DETERMINED.

Experiment details		Reduction in mortality %	
Salinity (PSU)	Concentration (mg/L)	"After-effect"	After 7 days
10	3	0	
	5	0	
	10	0	
	20	0	
	50	0	0
30	3	0	
	5	0	
	10	0	
	20	0	
	50	0.3	0

6.3.3 Ecotoxicological tests

Due to the lack of scientific references on Mexel®, several concentrations were tested ranging from 50 mg/L which is the recommended concentration by manufacturer to 0,01 mg/L. Moreover 1, 2, 3 weeks-old prepared solutions were used to check the degradability of the biocide.

All tested concentrations have no or low impact (between 2 and 20 % of abnormal larvae) on larvae development (Figures 6.3.3.1 and 6.3.3.2). Additionally the older the solution the higher the abnormal larvae percent increases (Figure 6.3.3.1). Considering this effect, albeit minimal, the recommendation could be the release of the treated water before 3 weeks. Finally toxicity of Mexel® is quite similar regarding the sensitivity of individuals (Figure 6.3.3.1, compare dark and dotted bars).

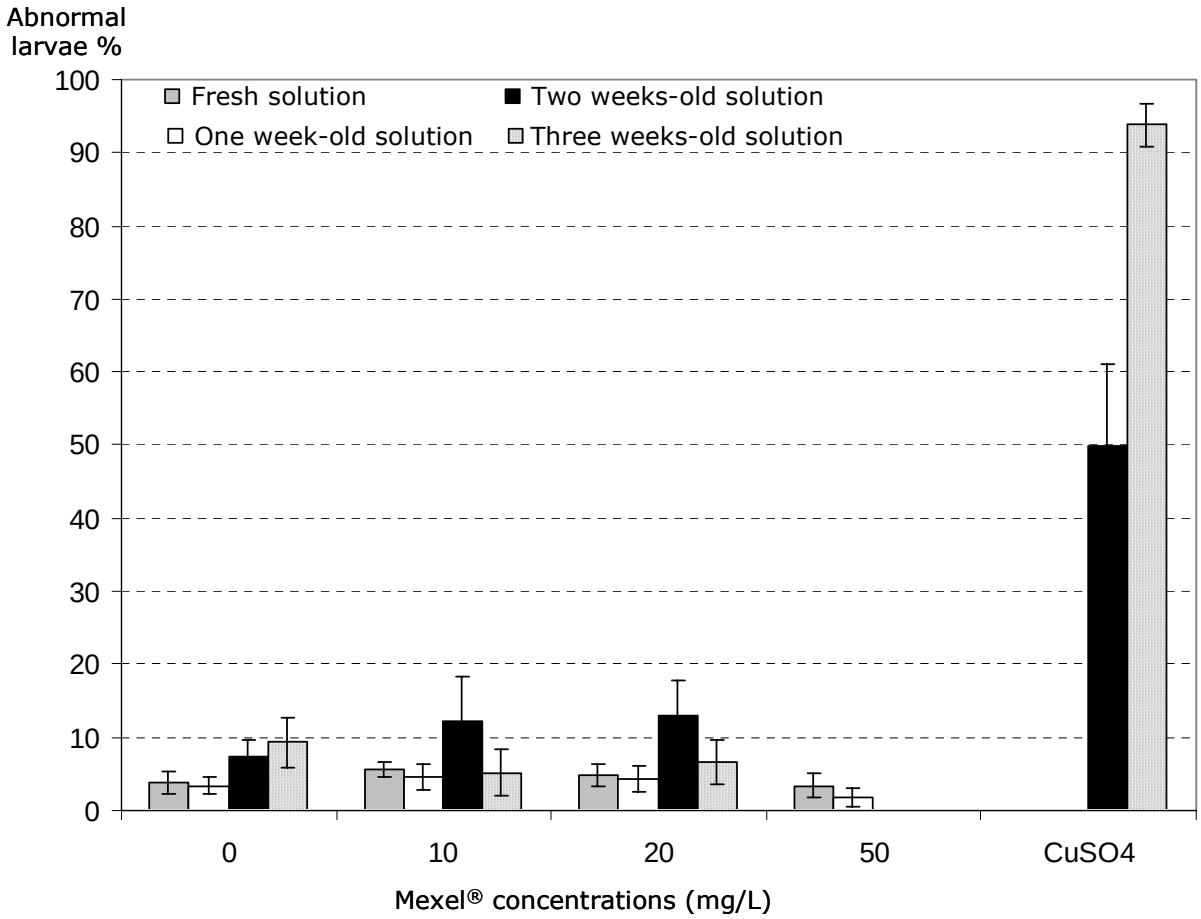


FIGURE 6.3.3.1 ECOTOXICOLOGICAL EFFECTS OF MEXEL® AFTER 24 HOURS OF INCUBATION RELATIVE TO SOLUTION "OLDNESS" AND TO SENSITIVITY OF INDIVIDUALS. Oyster larvae were exposed to 50, 20 and 10 mg/L of biocide or to 25 µg/L of $CuSO_4$ and abnormal larvae at the "D-stage" were count. Biocide concentrations were prepared from fresh (grey bars), one week-old (white bars), two weeks-old (black bars) and three weeks-old (dotted bars) solutions. *Abnormal larvae percent are mean values of five independent experiments and standard deviations are indicated. Two to four independent assays are represented.*

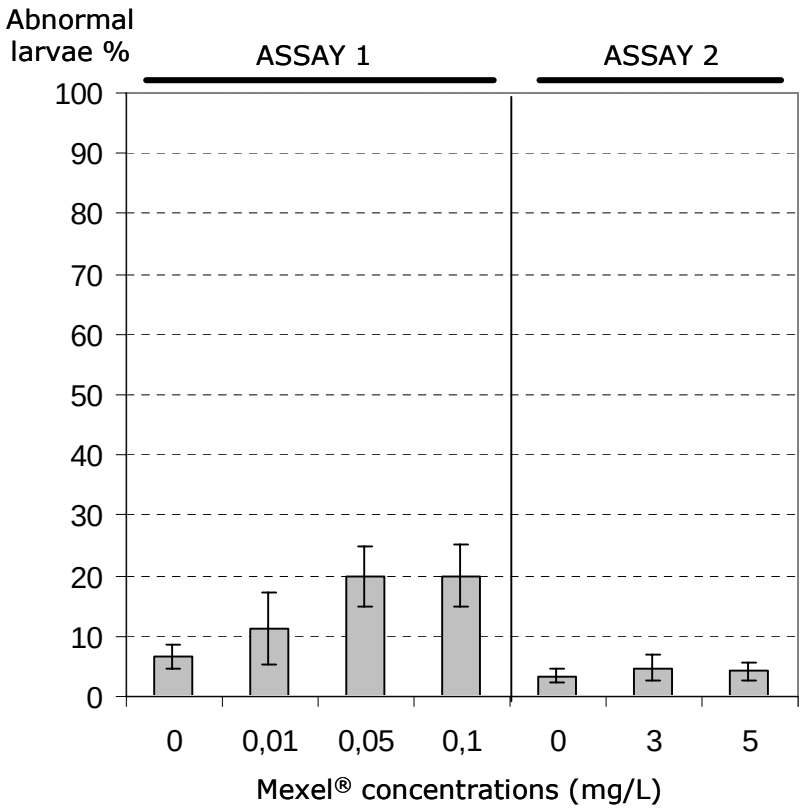


FIGURE 6.3.3.2 ECOTOXICOLOGICAL EFFECTS OF MEXEL® AFTER 24 HOURS OF INCUBATION. Oyster larvae were exposed to various biocide concentrations in two independent assays and abnormal larvae at the "D-stage" were count. *Abnormal larvae percent are mean values of five independent experiments and standard deviations are indicated.*

6.3.4 Preliminary conclusions on environmental acceptability of alkylamine based biocide

On one hand, degradation as well as decrease of toxicity of Mexel® was observed in bacteria tests in 24 hours. On the other hand, although some degradation of Mexel® was documented in *Artemia* bioassays, this did not result in a reduction of mortality rates. This indicates that effects of alkylamines depend on the organism considered.
In spite of this, concentrations from 0,01 mg/L to 50 mg/L have no impact on oyster larvae development in this study suggesting that the release of this biocide in the environment is acceptable.

6.4 Conclusions on environmental acceptability of biocides

Firstly, "after-effect" experiments on bacteria showed that PeraClean® Ocean and Mexel® display degradation in 24 hours and a decrease of toxicity. However it is important to note that the loss of toxicity is really poor in the case of high concentrations of Mexel® (15,5 mg/L, 1 to 3 % of survival).

Secondly, bioassays with *Artemia* showed that all three biocides had relatively poor degradation properties. None of the biocides showed notable reduction in efficiency after the initial 24 h, documented by mortality rates >92% in all cases. In the bioassays with PeraClean® Ocean and Mexel®, the mortality at the highest concentrations remained >98% even after 7 days, while the mortality with SeaKleen® was >84% at the same time point. For some biocides, toxicity appears to increase with time if stored in darkness. This was the case with the powder-like SeaKleen® (Raikow *et al.*, 2006) and with Mexel® in this study (see 6.4.3 section). Indications of increased toxicity against *Artemia* after storage in darkness was found for PeraClean® Ocean and Mexel®, and also SeaKleen® showed temporal increase in efficiency in the course of the 28 days incubation. On the other hand, a biocide may lose toxicity when in contact with live organisms, as was suggested for the powder-like SeaKleen® (Cutler *et al.*, 2004). In the *Artemia* bioassays, the overall density of live organisms was rather low (~20 ind./L), but it's not evident whether degradation could occur more rapidly in waters containing a higher abundance of living tissue.

Thirdly, ecotoxicological tests highlight Mexel® which is the only biocide displaying no impact on oyster larvae development whatever the concentration (from 0,01 to 50 mg/L).

7 CONCLUSIONS AND OUTLOOKS

Results of the laboratory tests are summarized in the following tables (Table 7.1 and 7.2).

TABLE 7.1 EFFICIENT BIOCIDE CONCENTRATIONS ON EACH ORGANISMS.

ORGANISMS	PERACLEAN® OCEAN	SEAKLEEN®	MEXEL®	OBSERVED PARAMETERS
PHYTOPLANKTON	100 – 200 mg/L	2 – 10 mg/L	50 mg/L	No growth during 4/5 days
BACTERIA	20 mg/L	> 500 mg/L	50 mg/L	LC90 in 24 h
ZOOPLANKTON	300 – 500 mg/L	5 – 6 mg/L	20 – 30 mg/L	

TABLE 7.2 ENVIRONMENTAL ACCEPTABILITY OF THE BIOCIDES, ASSESSED BY MEANS OF CHANGE IN TOXICITY DURING A 24-H RETENTION PERIOD (AFTER EFFECT TESTS) AS WELL AS OYSTER LARVAE TESTS.

ORGANISMS		PERACLEAN® OCEAN	SEAKLEEN®	MEXEL®	OBSERVED PARAMETERS
BACTERIA[1]	10 PSU	100 to 0 %	18 to 0 %	100 to 0 %	Change in mortality from the "effect" to "after-effect" tests [2]
	30 PSU	100 to 0 %	70 to 8 %	52 to 0%	
ZOOPLANKTON	10 PSU	96 to 94 %	95 to 93 %	96 to 99 %	
	30 PSU	100 to 100 % (no change)	97 to 95 %	100 to 99 %	
OYSTER LARVAE (ECOTOXICITY)		≤ 0,001 mg/L	< 0,005 mg/L	≤ 50 mg/L	Non-toxic doses (no abnormal larvae in 24h)

[1] The mortality percent varies regarding the strain so the strain displaying the biggest reduction in mortality between the "effect" and "after-effect" assays is indicated.

[2] Only results for the highest tested concentration are represented.

This study suggests that alkylamine based biocide (Mexel® 432/336) seems to be a good candidate for the next steps of the project consisting in large scale assays.

Firstly, regarding the criteria we fixed, it is efficient on all tested organisms (bacteria, phyto- and zooplankton) at concentrations ≤ 50 mg/L in 24 hours (Table 7.1).

Secondly, in the ecotoxicological tests, this substance seems to be non-toxic for concentrations below or equal to 50 mg/L (Table 9-2). Although the "after-effect" experiments on bacteria are satisfactory (not on all tested bacteria), the increased toxicity against *Artemia* after 24 hours is a matter of concern (Table 7.2).

Thirdly, Mexel® 432/336 is derivated from Mexel® 432/0 which is already successfully used in cooling water treatment of industrial installations. Consequently the formulation that we used meets the criteria of safe handling and probably of cost and displays the same properties than the original substance such as liquefying effects on sludge, action on biofilms and anticorrosion effects. These are really interesting in the ballast tank context. Moreover, Mexel® 432/336 is in accordance with the Directive 98/8/EC of the European Parliament and the Council of February 16, 1998 concerning biocidal products.

Finally, Mexel® 432/336 has never been tested as a ballast water treatment and in that way is of great interest.

Short-term perspectives (next chapter) are to confirm the laboratory tests results on a larger scale. This will be performed in a pilot system (Ballastodrome, see next chapter) on an "EFFORTS soup" which will consist in a mix of all organisms independently tested in the laboratory test phases. The aim of these experiments is to check if larger volumes influence Mexel® 432/336 efficiency. Moreover toxicity of the biocide and its degradation level after 24 hours will be re-evaluated, considering that the presence of several organisms could accelerate loss of toxicity of the biocide (Cutler *et al.*, 2004). This may counteract the observed increase in toxicity during storage in the *Artemia* bioassays (paragraph 6.3.2).

The longer-term prospects are to test this biocide in the ballast tank context to validate the efficiency and feasibility of this potential ballast water treatment (chapter 8).

8 SPECIFICATIONS AND OPTIMISATION OF LABORATORY LARGE SCALE TEST: "BALLASTODROME" DEVICE

8.1 Optimisation of "Ballastodrome" experimental device

For a ballast water treatment designer, building a real scale prototype can be very expensive. The laboratory experiments for treatment efficiency assessment are not sufficient to give all the information needed, and moreover they are often very far from real conditions aboard a ship. Therefore, it may be wise to test systems at an intermediate stage, thus sparing time and money. This led to the idea of a ballast reduced scale system, intermediate stage between laboratory and ship.

The pilot used here was built on naval grade steel like a real ship part, with girders and framing just like inside a ship's ballast tank, even epoxy-tar coated like the real situation.

The two 250 litre tanks (one can be used as control or different treatment test) are pivot mounted to simulate lurch. Several connections on each tank are fitted to put probes, take samples or connect the pilot to a reduced scale treatment circuit. The pilot is normally working in the dark (like aboard ships), the observation portholes covered in black.

FIGURE 8.1 Ballastodrome devise, marine station of Luc sur mer (France).

The only unavoidable difficulty is the relatively small size, comparatively to American or Norwegian systems which seem having several hundred cubic metres. On the other hand this one allows an easy handling, being lorry mounted, permitting light truck transportation to different sites. The use of this pilot can help to detect problems not obvious with laboratory glass vials assessment methods.

8.2 "EFFORTS soup" specifications and experimental procedure

Efficiency of the biocide Mexel® 432/336 is tested in two different kinds of experiments in the ballastodrome system: the first one on an "EFFORTS soup" and the second one on natural populations of turbid seawater from the English Channel.
The "EFFORTS soup" consists in a mixing of organisms species like it was the case for the "MARTOB soup" designed under the FP5 MARTOB program. The "EFFORTS soup" includes organism species used in the laboratory assays except for one phytoplanktonic species:

- 3 bacteria species: *Enterococcus faecalis*, *Aeromonas caviae* and *Vibrio vulnificus;*
- 2 phytoplanktonic species: *Tetraselmis suecica* and *Heterocapsa sp* which is a dinoflagellate very similar in size and behaviour to *Alexandrium* toxin producing species but no dangerous to use in such unprotected area;
- 1 zooplanktonic species: *Artemia sp.*

These organisms are introduced in 250L of 2 µm filtered seawater at a rate of 10^5 cells/ml for bacteria, 4 to 6.10^6 cells/L for phytoplankton and 100 ind./L for *Artemia* (MEPC125(53), 2005). Only the natural salinity of the English Channel seawater (between 30 and 34 PSU) is used since 10 PSU is not suitable for growth of marine phytoplanktonic species. Moreover no important differences in biocide toxicity between 10 and 30 PSU were observed in bacterial and zooplanktonic laboratory tests (see chapter 5.4 and 5.6). The seawater is maintained around 17°C and in darkness in the tanks.

A sample is taken before inoculation of the soup to determine the number of total viable bacteria naturally present in the seawater. After inoculation samples are also collected to check the number of each organism at the beginning of the experiment and Mexel® is added in one tank at 50 mg/L.

After 24 hours of incubation, the survival and the mortality rates are determined respectively for bacteria (total bacteria) and *Artemia* in each tank. Growth of phytoplanktonic species is followed for 5 days after addition of Mexel® compared to the control. Finally ecotoxicological tests are realized with seawater of the control and the treated tanks.

In the second set of experiments, the effect of Mexel® on natural communities of seawater was tested. The two tanks of the ballastodrome are filled with turbid seawater and the numbers of bacteria and phytoplanktonic cells and zooplanktonic individuals on day 0 are established. Moreover the suspended matter was defined. Then Mexel® was added in one tank at 50 mg/L and incubation was performed at 17°C in darkness for 24 hours. On day 1 samples are collected and studied for each organism type as described above. These last experiments are meaningful only if the seawater contains enough organisms.

9 WORK PROGRESS OF FULL/LARGE SCALE TEST TRIALS: ON-BOARD TESTS

The onboard test schedule is in progress.

A first meeting with a ship-owner is planned on March, and the aim is to perform the on-board tests between April and June 2009.

The objective is to define the onboard technical feasibility, and detailed specifications regarding:

- The active substance storage onboard and injection into the ballast water pipeline before ballast pumps.
- The experimental plan (required concentration, time of exposure, degrading time...).
- The sampling method.
- The treatment of samples, realised onboard (preparation of the samples before sending them to specialised laboratories).

According to the technical, financial and time-related possibilities, a second onboard test may be organized.

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