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USING HEAT TREATMENT OF BALLAST WATER FOR KILLING MARINE MICROORGANISMS

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Abstract: *By ships ballast water are transferred and discharged non-indigenous aquatic organisms into receiving waters. The Black Sea also was involved in such events as a source of organisms and as a colonized space too. To avoid that, the International Maritime Organization recommends treating the ballast water by means of different methods. One of them is based on heating ballast water in order to kill the organisms and microorganisms.*
The paper present a system for recovering the heat from main engine for rise the temperature of ballast water and the impact of different stages of heating on the total number of cells. The samples were collected from Black Sea, analyzed using epifluorescent microscope and to estimate the total number of cells the CellC software was used.
Keywords: *heating ballast water, marine microorganisms, epifluorescent microscopy*

1. INTRODUCTION

Ships use ballast water to provide stability and maneuverability during a voyage. In this process, there are transferred millions of tons of ballast water from one place to another.

The impact of non-native species introduced via ships' ballast water has been documented (Ruiz et al., 1997; Gollasch, 2002; Hewitt et al., 2004) because of their negative disasters created in the economic field, in the marine environment and also in people health; over $5 billion were losses due to invasion by the Zebra mussel in the Great Lakes in 1980 (Carlton, 2000 Tzankova, 2000), a comb jelly from U.S. Mnemiopsis leidyi was introduced in the Black Sea (1982) and preyed on fish larvae as well as their prey food, essentially wiping out the anchovy fishery there. In order to solve this problem the International Maritime Organization has elaborated an International Convention for the Control and Management of Ships' Ballast Water and Sediments (IMO, 2004). The Convention sets the number of viable organisms and microorganisms Table 1, allowed to be discharged with ballast water (IMO, 2005).

Species	Concentration
Toxicogenic Vibrio cholerae	Less than 1cfu per 100ml
Escherichia coli	Less than 250cfu per 100ml
Intestinal Enterococci	Less than 100cfu per 100ml

Tab.1. The indicator microbes for ballast water

The heat treatment of ballast water has been presented as a possible treatment method based on theoretical (Bolch and Hallegraeff, 1993; Hallegraeff et al., 1997; Mountfort et al., 1999) and laboratory trials (Rigby et al., 1999; Mountfort et al., 2001). Different methods of heating the ballast water on board vessels have been proposed (Rigby and Hallegraeff, 1994; Rigby et al., 1997; Mountfort et al., 2001; Quilez-Badia G. et al. 2008).

Most of the experiments until now were focused on different species of marine organisms (Bolch and Hallegraff, 1997; Montani et al.1995), table 2.

Species	Temp.	Time	Source
	°C	hms	
G. catenatum	35-37.5	1-2h	Hallegraeff et al. (1997)
G. catenatum	40-45	30-90s	Bolch and Hallegraeff (1993)
Alexandrium Sp.	45	3m	Montani et al (1995)
A. cattenella	38	4h30m	Hallegraeff et al. (1997)

Tab. 2. Lethal temperature for different species

We counted the total number of microorganism and the evolution at different stages of heating.

2. BALLAST WATER TREATMENT BY HEATING

Ballast water is heated by using the engine cooling system. This is a very efficient method because coolant water is necessary and would most likely be discharged into the ocean if it were not used to treat ballast water. Another benefit to this method is that there are no resulting chemical byproducts.

2.1 The retrofitting of the ballast system

Ballast water is heated by using the engine cooling system.

The first modification of the ballast system is to use as ballast the sea water that was used as a coolant agent for the main engine low temperature cooling system is usually discharged overboard. We use this water for filling the ballast tank with sea water at 25°C as result from the diagram.

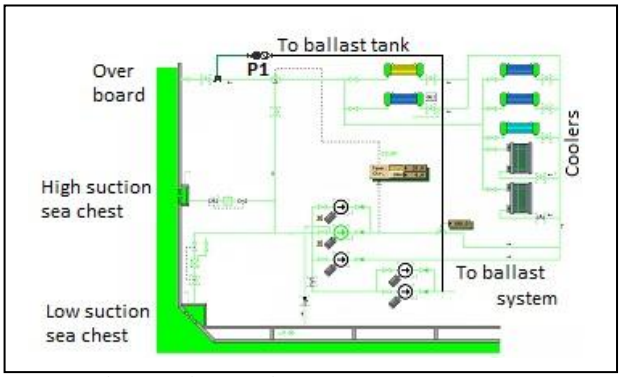

Fig. 1. The first modification of the ballast system

The temperature is not enough to kill the microorganism; so we will use a 20 percent of the flow from high temperature main engine cooling system at 80°C temperature. This flow rate is directed by a heat exchanger that will heat the sea water from the ballast tank.

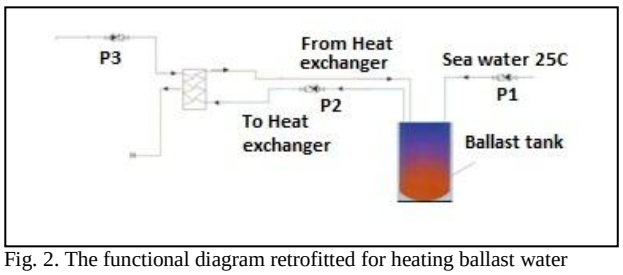

Fig. 2. The functional diagram retrofitted for heating ballast water

This is a very efficient method because coolant water is necessary and would most likely be discharged into the ocean if it were not used to treat ballast water. Another benefit to this method is that there are no resulting chemical byproducts.

The treatment method is limited by the amount of heat provided by the engines, so the amount of ballast water to be treated must be compared to the heat released by the engines. A number of factors need to be considered before using heat treatment on a specific ship or a particular voyage. The voyage must be long enough to allow water to reach the specified temperatures for the necessary amount of time.

2.2 The methodology used for analyzing the samples

The most used methods for estimate the viable/ death cells based on colorful substances are those screening the entire membrane of the cells. These are made visible by coloring with fluorine components.

Sample collection The sea water samples were collected from Black Sea area, latitude 44º07´.5 N and longitude 028º41´.7 E, in June 2012, 9 UTC, air temperature 27°C and sea water temperature 25°. The samples were screened in Marine Environment Laboratory from Constanta Maritime University. There were three samples 2l each, collected in bottles.

Fluorescence microscopy is based upon the concept that there are certain materials that emit energy which can be detected as a visible light. Each of these materials must be irradiated with a different specific light wavelength in order to cause an energy reaction in the form of light. The sample being used can either be treated with some fluorescing substances or it can be fluorescing on its original form. Fluorescence microscopy differs from most traditional techniques in that the visible light in the microscope eyepieces is not the original light emitted by the light source. The light seen is actually light that has fluoresced from the specimen itself. In order to receive such a response a high intensity light source must be used. This light is passed through a dichroic filter cube containing a fluorescence band pass excitation filter, which only allows specific wavelengths of light to pass and reach the fluorescence specimen. After the incident filtered light reaches the specimen, it is no longer used, and any amount reflecting back into the microscope objective to the dichroic mirror is refined by the emission filter. The specimen fluoresces and it is this fluorescing light that passes back through the fluorescence emission filter and goes to the microscope eyepieces to provide a bright and colorful fluorescence image of the specimen.

Epifluorescent microscope utilizes a 100W mercury burner light source. The epifluorescence microscope features a ocular head; 4x, 10x, 40xR and 100xR (oil) planachromatic objectives; a blue (450-480 nm) /green (510-550 nm) dual band filter set; The filter sets provide superior performance in conjunction with a wide variety of most common fluorophores. The 40xR and 100xR objectives feature a retractable lens.

Samples were viewed immediately by epifluorescence microscopy (N-400FL, lamp Hg 100 W, type on the blue filter- 450-480 nm) with immersion 100X objective and 10X eyepieces; AO staining (10 µg/mL); Measurement of 150 cells / filter.


Fig. 3. The epifluorescent microscope

The main advantages of epifluorescent microscopy is that microorganism detection no need to much time, the analyses are made in real time and the fluorochromes is used diluted (a good cost to quality ratio). The disadvantages could be represented by the necessity of a special quartz light source.

Colorant epifluorescence SYBR-Green I (1000 µg/mL), is a small molecula that could penetrate the viable cells and also the death cells giving them a green fluorescent light. Reported previously for use with flow cytometry (Marie el al. 1997), SYBR Green I apears to be a viable tool for enumerating viruses and bacteria in seawater.

SYBER Green is an asimetric cyanine stain used for staining the nucleic acid in molecular biology. It has the advantage that is inexpensive and the manufacturer claims it to be much less carcinogenic (Noble T, 1998) than other typical nucleic acid stains.

For the total cell count we used two fluorochrome (SYBR Green I) in final concentration 10 µL/mL, 1: 100.000 (SYBR Green I), and staining time – 10 minute. The protocol work followed in agreement with (Lebaron et al., 2001; Sherr et al., 2001; Paul, 2001; Ardelean et al., 2009; Ghita et al., 2010; Ghita and Ardelean, 2010).

Other materials: filter membrane Millipore de 0.22µm, Sudan black, Millipore glass filter holder.

2.3 The method used for counting the cells

CellC Software can be used in automated analysis of microscopy images for counting the cells or for measurements of cell proprieties. We used it for counting the cells. The binarized results images are saved as JPG and the enumeration result ans statistic are saved as an Excel-ready CSV file.

For each filter we screen 20 fields, and using CellC: Software for quantification of labeled bacteria by automated image analysis based on MATLAB, we obtained the number of cells on analyzed filters.

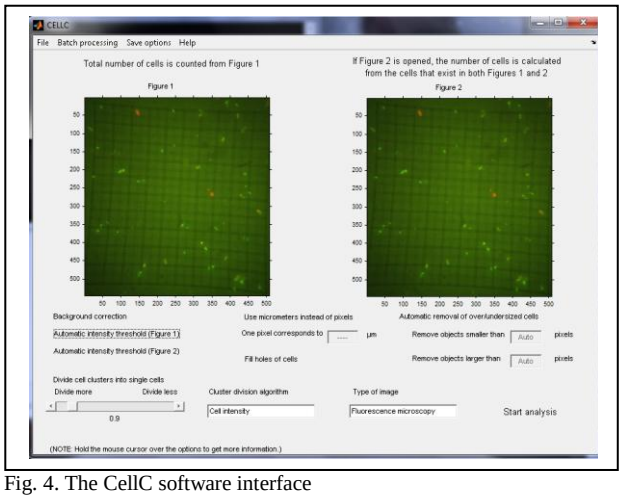

Fig. 4. The CellC software interface

In order to obtain accuracy results, the visualisation of the screened field is very important. It could be necessary to eliminate the "dirt". The imagines also should be taken with the same photo camera at the same rezolurion to avoid false final results.

2.4 The experiment

The sea water sample 10ml was mixed with 10µL SYBR Green I /ml sample, final concentration. The final concentration of SYBR Green I, used for counting the number of cells from each sample, was obtained with a dilution 1: 100.000 with de 10µL SYBR Green I /mL sample.

After 5 minutes, the mixing (sample + stain) was filtered by a Millipore membrane, using a syringe and the holder. The filters used for the experiment were Millipore membrane filter polycarbonate 0,22µm, d= 47mm, that retain most of the microorganisms. For blackening the filters we used Sudan black 0,1g + 30mL ethanol 70% in order to minimize the background fluorescence.

After filtering process, supra stain was removed by washing with 10-20ml sterile water. Each filter was laid-down on a glass slide and analyzed using the Epi-fluorescent Microscope N-400FL, by blue filter (450-480 nm) for current fluorochrome (SYBR Green I), having 100W mercury burner light source, magnification 1000x (objective for immersion 100x, and grid ocular 10x), with one grid ocular micrometer, calibrated before.

We use the software CellC for counting (for automated image analysis, CellC being the main software used for processing multiple digital microscope images for count cells (http://www.cs.tut.fi/sgn/csb/cellc/).

The number of cells was transformed using the Fry, 1990 equation in number of cells per ml, by a ocular grid calibrated for microscope type (0,01mm^2 aria).

$$T = \frac{NA_f}{aV} \quad (1)$$

where, T - number of cells per volume unit, N - number of cells per grid aria, A_f - filtering aria,
V - volume of the filtered sample and a - grid aria.
For each filter was screened 20 fields (recommended by Gough & Stahl, 2003).

Cell's serial number	Exists in Figure2 (YES=1, NO=0)	Area of cell	Approximate volume	Length	Width	Intensity mean	Intensity maximum	Solidity	Compactness
1	1	6.693	23.392	1.647	1.647	53.25	58	1	52.567
2	1	6.693	23.392	1.647	1.647	71.5	83	1	52.567
3	1	83.663	45.688	20.587	20.587	71.2	81	1	42.054
4	1	250.989	486.488	45.292	45.292	812.667	133	0.88235	26.066
5	1	100.396	78.949	24.705	24.705	633.333	84	1	35.045
6	1	167.326	187.139	3.294	3.294	66	80	1	32.854
7	1	50.198	0.98686	12.352	12.352	653.333	68	1	70.089
8	1	6.693	23.392	1.647	1.647	63.25	70	1	52.567
9	1	284.454	486.488	45.292	45.292	860.588	136	0.94444	29.542
10	1	33.465	0.2924	0.8235	0.8235	66.5	67	1	105.134
11	1	234.256	266.454	37.057	37.057	764.286	91	1	36.343
12	1	234.256	365.506	41.175	41.175	90.5	133	1	29.438
13	1	33.465	0.2924	0.8235	0.8235	58.5	61	1	105.134
14	1	50.198	0.98686	12.352	12.352	63	68	1	70.089
15	1	100.396	45.688	20.587	20.587	85.5	122	1	50.464
16	1	200.791	365.506	41.175	41.175	81.5	106	1	25.232
17	1	50.198	0.98686	12.352	12.352	77	81	1	70.089
18	1	83.663	45.688	20.587	20.587	47.2	50	1	42.054
19	1	6.693	23.392	1.647	1.647	58.25	62	1	52.567
20	1	284.454	486.488	45.292	45.292	907.059	168	1	29.542
21	1	16.733	0.036551	0.41175	0.41175	61	61	1	210.268
22	1	33.465	0.2924	0.8235	0.8235	70	73	1	105.134
23	1	33.465	0.2924	0.8235	0.8235	69.5	79	1	105.134
24	1	83.663	45.688	20.587	20.587	88.8	101	1	42.054
25	1	6.693	23.392	1.647	1.647	63	70	1	52.567
26	1	167.326	187.139	3.294	3.294	78.1	119	1	32.854
27	1	184.059	266.454	37.057	37.057	774.545	108	1	28.555
28	1	317.919	803.016	53.527	53.527	706.842	132	0.95	2.364
29	1	33.465	0.2924	0.8235	0.8235	79	81	1	105.134
30	1	83.663	45.688	20.587	20.587	63.2	65	1	42.054
31	1	133.861	125.368	28.822	28.822	86.75	120	1	34.329
32	1	267.722	631.594	4.941	4.941	99.75	180	0.88889	23.363
33	1	50.198	0.98686	12.352	12.352	646.667	72	1	70.089
34	1	50.198	0.98686	12.352	12.352	533.333	56	1	70.089
35	1	50.198	0.98686	12.352	12.352	646.667	71	1	70.089
Statistical means of columns									
0	0	11.665	14.732	23.411	23.411	716.052	911.429	0.99045	57.851
Unit of measure: pixels									

Fig. 5. CellC number

The fields were selected randomly in order to catch different cells density. The images were taken using digital photo camera Sony 7.2 megapixels.

3. THE RESULTS

We presented here the microorganism evolution when the ballast water was heated at 35°, 40°C and 45°C. We obtained the total number of viable cells in sea water, normal condition of temperature 25°C, presented in the 2nd column and the total number of viable cells after heating at T_i temperature for different periods of time t_1, t_2 and t_3. We also mentioned the SD - standard deviation which shows us the difference between the screened samples for each field.

Sample no.1

Total no. of cells	Initial cond. T_o = 25°C	Constant temperature T_1 = 35°C		
		T_1-t_1 after 12 hours	T_1-t_2 after 48 hours	T_1-t_3 after 96 hours
media x10^6	12.03	6.56	3.51	2.16
±SD	3.4	1.45	0.68	0.27

Tab. 3. The number of cells after heating at 35°C

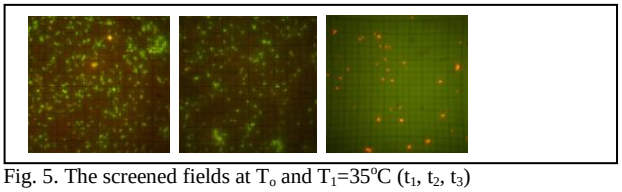

Fig. 5. The screened fields at T_o and T_1=35°C (t_1, t_2, t_3)

Sample no. 2

Total no. of cells	Initial cond. T_o = 25ºC	Constant temperature T_2= 40ºC		
		T_2-t_1 after 12 hours	T_2-t_2 after 24 hours	T_2-t_3 after 36 hours
media x10^6	10.84	5.57	2.79	1.23
±SD	2.67	1.23	0.98	0.42

Tab. 3. The number of cells after heating at 35ºC

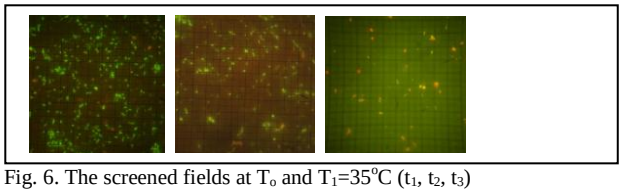

Fig. 6. The screened fields at T_o and T_1=35ºC (t_1, t_2, t_3)

Sample no. 3

Total no. of cells	Initial cond. T_o = 25ºC	Constant temperature T_3= 45ºC		
		T_3-t_1 after 1 hour	T_3-t_2 after 2 hours	T_3-t_3 after 4 hours
media x10^6	11.67	3.12	1.06	0.64
±SD	2.92	0.55	0.19	0.31

Tab. 3. The number of cells after heating at 35ºC

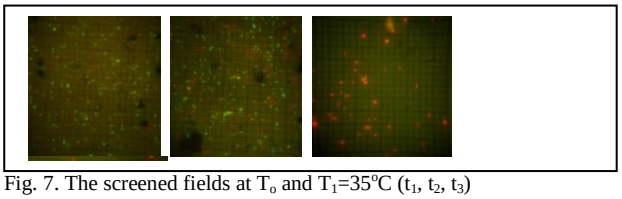

Fig. 7. The screened fields at T_o and T_1=35ºC (t_1, t_2, t_3)

4. CONCLUSIONS

The laborator experiments results shown that keeping ballast water for four days at 35ºC , kills 80% of the microorganism, one day and a half at 40ºC, kills 90%. Those percents are inacceptable for treating ballast water in naval transport because International Maritime Organisation set rulles regarding the desinfection by the Convention for of ballast water management and Sediments. The eficacity in killing the microorganism is aproximatly 95% if we keep for four hours the temperature at 45ºC. This percent is a good one but the method should be applied on a large scale, for a ship and based on the results the International Maritime Organisation would certify the method.

For retrofitting the ballast water system we considered that only one single ballast tank is suitable for heating. So, after the treatment method was applied, the treated ballast water should be moved from this tank to another one. That way a new quantity of sea water would be filled in the same tank and the heating treatment would start again.

Because of this period of delay, the treatment method of ballast water by heating is suitable for those types of ships that have small quantities of ballast water to be treated, for example port-containers and general cargos, rather than the large ships like petroleum tanks that unload all the cargos once and need to fill most of the ballast tanks with sea water, almost at the same time.

This analyze was based on the total number of microorganisms and their evolution when the sea water was heated in stages; the future research activity of the authors will be based on identifying the species of microorganisms from sea water, sampled from Black Sea, and their evolution during the heating process.

On the other hands we will work for optimizing the costs for retrofitting the ballast water system in order to install the above mentioned heat exchanger.

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