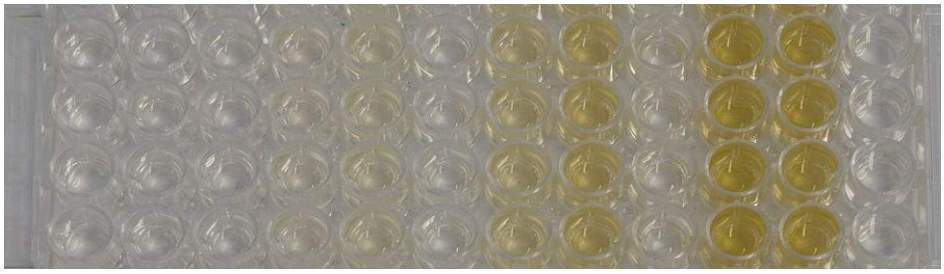
The effect of low temperatures on the toxicity of PERACLEAN® Ocean

N.H.B.M. Kaag & A.C. Sneekes

Report number C088/13



IMARES Wageningen UR

(IMARES - Institute for Marine Resources & Ecosystem Studies)

Client: Evonik Industries AG
Rodenbacher Chaussee 4
63457 Hanau-Wolfgang
Germany

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P.O. Box 68
1970 AB IJmuiden
Phone: +31 (0)317 48 09 00
Fax: +31 (0)317 48 73 26
E-Mail: imares@wur.nl
www.imares.wur.nl

P.O. Box 77
4400 AB Yerseke
Phone: +31 (0)317 48 09 00
Fax: +31 (0)317 48 73 59
E-Mail: imares@wur.nl
www.imares.wur.nl

P.O. Box 57
1780 AB Den Helder
Phone: +31 (0)317 48 09 00
Fax: +31 (0)223 63 06 87
E-Mail: imares@wur.nl
www.imares.wur.nl

P.O. Box 167
1790 AD Den Burg Texel
Phone: +31 (0)317 48 09 00
Fax: +31 (0)317 48 73 62
E-Mail: imares@wur.nl
www.imares.wur.nl

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Contents

Summary

Ballast water treatment systems are routinely tested for efficacy and residual toxicity at spring and summer temperatures in temperate areas, as that is the period when biological production is sufficiently high to match IMO testing requirements (guideline G8). Shipping, however, continues throughout the year and, as polar ice rapidly disappears, also shipping into the Arctic rapidly increases. This makes assessment of the performance of ballast water treatment technology at low temperatures urgent, especially when biocides are used.
In order to assess the influence of temperature on the efficacy (toxicity) of PERACLEAN® Ocean (PO), IMARES has tested the toxicity of (PO) to various organisms at standard test temperatures and at low temperatures.
It appeared very difficult to adapt standard test species to the extremely low temperatures needed (<5°C), and the results of the test with these species are unambiguous.
More progress was made using cultures of natural communities samples at the desired temperatures. These showed that at higher temperatures, the direct toxicity of PO increases by the addition of catalase, but that at low temperatures PO alone is just as toxic. An interesting observation is that at higher temperatures, the natural communities show some recovery (regrowth) after prolonged exposure (5 days), which was not observed at temperatures <5°C. The recovery was less when catalase was added to the treatment.

1. Introduction

Evonik Industries AG is developing procedures for the use of PERACLEAN® Ocean (PO) in Ballast Water Management Systems (BWMS). The prime active substance is peracetic acid (PAA). This compound degrades rapidly at higher ambient temperatures, but is more stable at low ambient temperatures. Organisms may also be less susceptible to active substances at low temperatures due to the lower speed of physiological processes, thereby compensating for the prolonged presence of PAA at low temperature. The net result of both processes is difficult to predict. Therefore, Evonik has asked IMARES to perform laboratory tests in order to assess the efficacy of PO with and without presence of catalase at low temperatures (< 10ºC, ideally ≤ 5ºC).

There are two situations where a ballast water treatment system has to cope with low water temperatures: during the winter in temperate areas and during the summer season in Arctic areas. Both situations are different, as the Arctic summer conditions represent the most productive season, whereas the temperate winter conditions represent the season where most organisms are dormant. It is, however, not clear whether this also implies structural differences in sensitivity. The scarce literature suggests that there is no consistent difference in sensitivity at the species level (e.g. Chapman *et al.* 2006). The sensitivity may, on the other hand, be different for species tested at their optimum temperature compared to species tested after adaptation to an extreme temperature (e.g. Camus *et al.* 2004).

In this research project, four approaches were followed:

1. Standard test organisms were adapted to lower temperatures and test results were compared with exposure at normal test temperature.
2. Antarctic algae (kindly provided by Dr.Timmermans of the Royal NIOZ) were cultured at 4°C for a direct assessment of the sensitivity of polar organisms.
3. Fresh water from the canal, and sea water from the harbour was collected in spring and further cultured at the laboratory at temperatures <10°C. The organisms developing in these cultures were tested for sensitivity.
4. Field samples were collected from October on every month. The sensitivity of these natural communities was tested at ambient temperatures at that moment.

Additionally, a literature survey was conducted in order to collect information on relative sensitivity of organisms at low temperatures.

This research is part of the strategic research program KBIV “Sustainable spatial development of ecosystems, landscapes, seas and regions" which is funded by the Dutch Ministry of Economic Affairs, and carried out by Wageningen University Research centre.

2. Background

Shipping activities have a direct effect on the rate of introductions of non-indigenous species (NIS) in an area (Gollasch 2006). In 2004 the IMO Ballast Water Convention was adopted, in order to prevent further spread of NIS by ship's ballast water. Consequently, ballast water management systems (BWMS) are being developed by companies all over the world. Before these BWMS may be used on board of ships, they need to obtain Type Approval by a flag state. In case the system is based upon the use of an active substance (toxicant), the BWMS also needs to be granted Final Approval by the GESAMP. In both cases, efficacy of the BWMS has to be tested to ensure that the system does reduce the number of organisms in the ballast water to such an extent that the criteria laid down in section D-2 of the Ballast Water Convention are met. Land-based testing is the most rigorous, as it is done on land under controlled conditions. During these tests, the challenge water needs to contain a minimum number of organisms (G8). Nearly all land-based test facilities are located in productive temperate areas. Consequently, most land-based testing is restricted to the productive season from approximately April to September, depending on location. When testing commences, the water temperature will generally be over 10°C. The remainder of the year, the surface water does not contain sufficient numbers of organisms to fulfil the requirements stated in IMO Guideline G8. The shipboard test may continue into the winter season as they do not require such stringent challenge conditions. Shipboard testing is, however, much less rigorous. This means that BWMS are usually not tested in water with low temperatures close to the freezing point. In real life, however, a BWMS may encounter low temperature challenge water, either when the vessel sails in temperate waters during the winter season, or when the vessel sails into Arctic (or Antarctic) waters. It is, therefore, necessary to know how a BWMS performs at low temperatures and –when considering a BWMS using an active substance- whether the concentration at discharge can be considered safe.

The Arctic

In the period 1980-2007, Arctic summer sea ice cover has decreased by 40%, while the volume even decreased by 70% (Humpert & Raspotnik 2012). In September 2012, the Arctic ice cover reached a new all-time low of 3.29 million km^2. This is 18% below the 2007 minimum and 49% below the 1979-2000 average (Arctic Sea Ice News & Analysis Sept 19th, 2012). This means that it will be possible to sail seasonally through Arctic waters without the aid of ice-breakers. Actually, in September 2009 when ice cover was 150% of the 2012 cover, two vessels belonging to Beluga Shipping GmbH sailed from Vladivostok to Rotterdam, using the Northern Sea Route (Reuters, Sept 12th 2009). Sailing through the Arctic is limited to the (late) summer season and may not be suitable for all types of ships due to the narrow and shallow straits that have to be passed on some routes (Humpert & Raspotnik 2012). The significant reduction in length of the voyage and the possibility to escape the dangerous areas near the Horn of Africa, make these routes attractive alternatives. Additionally, the accessibility of the area will boost Arctic offshore mining activities, especially for oil and gas exploration.

At the beginning of this millennium, 18 marine NIS were identified in European Arctic water (Gollasch 2006) and 10 in Alaska (Ruiz *et al.* 2006). Species extending their range northwards due to the increasing temperatures may reach the Arctic anyway. Shipping merely increases the speed of range extension. More important is species transfer within the Arctic or through the Arctic, thereby connecting the Pacific and Atlantic flora and fauna that have a completely different evolutionary history (Vermeij 2010).
The growing season in the Arctic is short and temperatures remain low because of the low intensity of the light. Due to the long days, however, the Arctic growing season is very productive. A BWMS, therefore, has to operate at temperature close to zero, while it may be challenged by large numbers of organisms.

3. Materials and Methods

Standard test organisms

Rotifers and microalgae were chosen as standard test organisms in this part of the research. PERACLEAN® Ocean was used as test substance, either direct (PO), or after pre-dosing of catalase (PC). For reference, the test species were exposed to a regularly used reference toxicant, potassium dichromate ($K_2Cr_2O_7$). Potassium dichromate is a stable compound, ensuring that the test concentrations are not different at different temperatures. There is an internal record of test results at the normal test temperatures.

Rotifers appear in natural water early in the season and were among the least sensitive species in previous tests with PERACLEAN® Ocean (Sneekes *et al.*, 2007a). Cysts of rotifers are commercially available for use in ecotoxicological research. The marine rotifer *Brachionus plicatilis* was used for tests with salt water, while *B. calyciflorus* was used for the freshwater tests. The general description of the rotifer tests is given in Appendix A. For the purpose of this research, the cysts were hatched at the required test temperature, so that the hatched larvae were adapted to that temperature.

Microalgae are relatively sensitive to PERACLEAN® Ocean (e.g. Sneekes *et al.* 2007b). At the laboratory, the marine species *Phaeodactylum tricornutum*, as well as the freshwater species *Pseudokirchneriella subcapitata* are kept in continuous cultures for the purpose of ecotoxicity testing. The general test procedures for the algae growth inhibition tests are given in Appendix A. For this research, cultures were set-up at different temperatures in order to adapt the algae to lower temperatures.

Antarctic algae

Chaetoceros dichaeta, C. brevis, C. debilis, Proboscia alata, Fragilariopsis kerguelensis and *Phaeocystis antarctica* were provided by Dr. K. Timmermans of the Royal NIOZ at Texel. These were cultured at 4°C, with a 16/8 h light/dark regime on f/2 nutrient medium. The cultures grew very slowly. During a power failure of the climate chamber during a heat wave, the cultures were lost.

Calibration studies have been executed with these species, in order to find the required settings for the equipment used. The Antarctic species have not been used for testing toxicity.

Culturing natural communities

Early May, natural water was collected from the Noordhollands kanaal (a freshwater canal) and in the harbour of Den Helder (sea water). The temperature of the water was still below 10°C. The intention was to collect the species that dominate the spring community as these are probably the most tolerant to low temperatures. The natural communities were roughly separated in a community >50µm and <50µm, by filtration through a 50µm net. The cultures were installed at the laboratory at a temperature of ca. 8°C, with a 16/8h light/dark regime. The <50µm community was supplemented with algae growth medium to stimulate phytoplankton development. The >50µm community was fed with algae in order to culture the zooplankton present.

Monitoring natural communities

In order to understand what happens in nature, water was sampled every 4 weeks at a freshwater location and at a salt water location. The samples were split in fractions <50µ and >50µm using a net. Both fractions were exposed to PO in different concentrations for 1 and 5 days. During the exposure period, the samples were stored in a climate chamber at temperatures equal to those measured at the field location during the sampling.

4. Results

Standard test organisms

The freshwater algae species *Pseudokirchneriella subcapitata* was not able to grow at 5°C. Indeed, this species can be stored at 5°C in the dark for years. As a result, also at 10°C growth was negligible and for as yet, no results have been obtained. At the standard test temperature of 23°C, adding catalase significantly reduced the toxicity of PO.

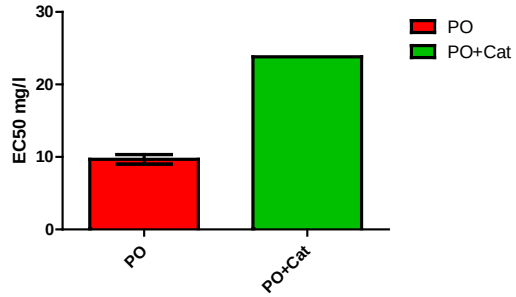

Figure 1 *EC_{50} for growth inhibition of the freshwater algae* P. subcapitata *at 23°C, exposed to PERACLEAN Ocean (PO) or PO + catalase.*

The marine algae *Phaeodactylum tricornutum* did grow at 5°C, but very slow. It took 5 to 10 days to attain the minimum required biomass increase (16x), which at 20° is reached in 72h and in 96h at 10°C. The prolonged exposure at 5°C seems to result in increased sensitivity (Figure 2) *P. tricornutum* was not exposed to PO without catalase.

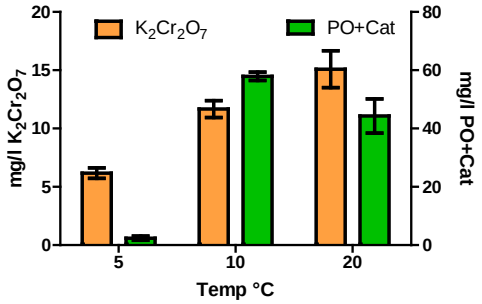

Figure 2 *EC50 for growth inhibition of the marine algae* P. tricornutum *exposed to$K_2Cr_2O_7$ (orange, left y-axis) and PO+catalase (green, right y-axis) at three different temperatures (exposure 48-96h at 10 and 20°C; 144-240 h at 5°C)*

Hatching time of the rotifers dramatically increased at low temperatures. The hatching was also less synchronised as it occurred over a longer period of time (Table 1). As a consequence small amounts of organisms were available for testing. If the amount was too small for a serious test, hatching of more cysts had to be awaited, resulting in a more heterogeneous age distribution. The freshwater species *B. calyciflorus* hatched very badly at 5°C. Only in one of the three attempts some cysts hatched.

Table 1 Hatching time for rotifers at different temperatures.

	25°C	10°C	5°C
B. plicatilis (marine)	24-30 h	5-6 d	10-14d
B. calyciflorus (fresh)	16-18 h	4 d	9d - ∞

Rotifers were exposed to PERACLEAN Ocean (PO) with and without addition of catalase. The results are shown in Figure 3.

For the marine species *B. plicatilis*, addition of catalase did not have significant effects on the toxicity (Anova, $p>0.05$), but at lower temperatures (5 or 10 °C) PO was significantly more toxic than at the standard test temperature of 25°C (Anova, $p<0.05$). For further analysis data for exposure to PO and PO+catalase are combined.

The effects on the freshwater rotifer *B. calyciflorus* were not significant (Anova, $p>0.05$), but are hampered by the fact that in 4 of the 5 tests with PO+catalase, the $EC_{50}$was higher than the highest concentration used (4.7 mg/l), while PO alone was only tested once at each temperature. The results suggest that PO+catalase is slightly less toxic, compared to PO alone.

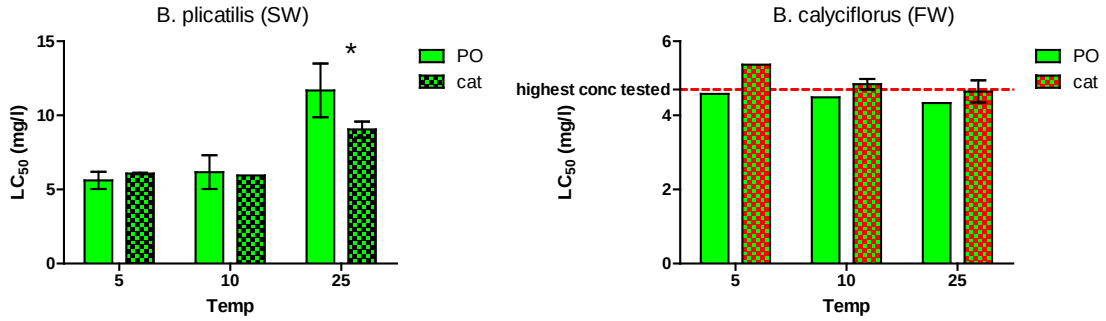

Figure 3 Results for the rotifers, tested with PERACLEAN Ocean (PO) and PO + catalase (cat). For the marine rotifer B. plicatilis*, the toxicity at 25°C is significantly less than at lower temperatures.*

As is shown in Figure 4, the effect of temperature on toxicity for the marine species *B. plicatilis*is not the same for $K_2Cr_2O_7$ and PO. For PO, toxicity at 5 and 10°C is the same ($p>0.05$) and significantly higher than at 25°C. For $K_2Cr_2O_7$, toxicity is the same at 5 and 25°C ($p>0.05$), but significantly lower at 10°C ($p<0.01$).

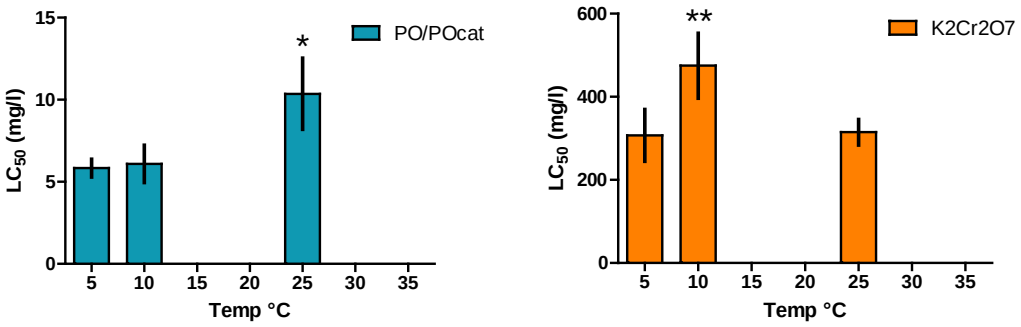

Figure 4 Effect of temperature on the toxicity of PERACLEAN Ocean (PO) and a metal ($K_2Cr_2O_7$) for the marine rotifer B. plicatilis*. For PO exposure also data for exposure with additional catalase are included.*

For the freshwater species *B. calyciflorus*, toxicity of both PO (without catalase), and $K_2Cr_2O_7$ seem to be lower at 5 and 10 °C, compared to the toxicity at 25°C (Figure 5). The effects can, however, not be tested due to lack of valid replicates.

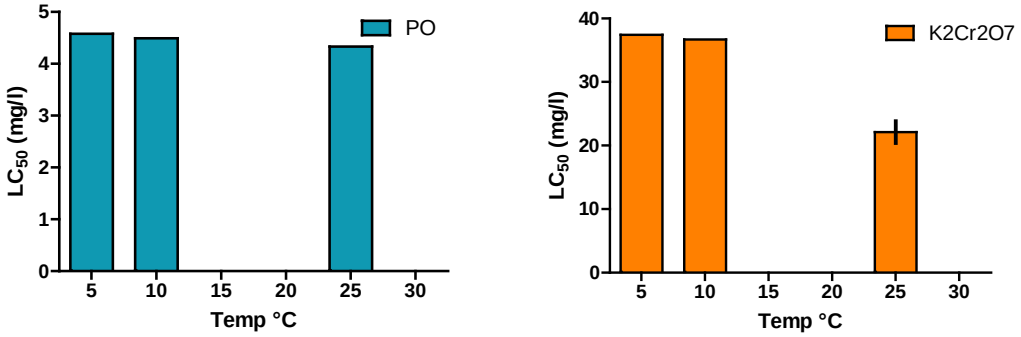

Figure 5 Effect of temperature on the toxicity of PERACLEAN Ocean (PO) and a metal ($K_2Cr_2O_7$) for the freshwater rotifer B. calyciflorus. For PO exposure also data for exposure with additional catalase are included.

Antarctic species

Six species of Antarctic algae were obtained from NIOZ. These were cultured at 4°C. Two species showed growth in the 96 microwell plates used for growth inhibition tests. For both *Chaetoceros debilis*, as *C. brevis* the growth rate was, however, still very slow. Both needed one week to obtain a 6-fold increase in cell density. The other species (*C. dichaeta, Proboscia alata, Fragilariopsis kerguelensis, Phaeocystis antarctica*) sustained in culture, but collapsed in test conditions. Due to technical failure, all cultures were lost during summer.

Cultured natural communities

The water collected in spring was dominated by phytoplankton. The freshwater community did not develop a standing stock at the low temperatures of the culture chamber. The phytoplankton in the marine community was dominated by *P. tricornutum*, the same species as is used as standard test organism. These were tested at 5°C and 10°C (Figure 6). The 'wild type' *P. tricornutum* appears to be slightly less sensitive than the cultured *P. tricornutum* that is used as standard test organism, but the

pattern is the same: comparable toxicity for $K_2Cr_2O_7$ at both temperatures, and much higher toxicity for PO(+cat) at 5°C.

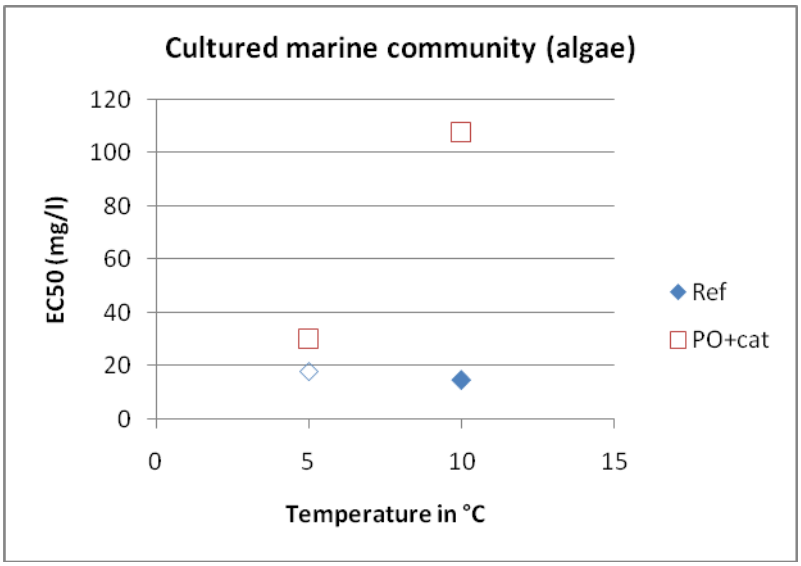

Figure 6 EC50 (mg/l) for a cultured marine algae community PO+catalase (red squares) and K2Cr2O7 (blue diamonds) at 5 and 10°C. Data for open symbols based upon only 1 test.

In the marine water cultured at different temperatures, ciliate populations developed on the decaying material at the bottom of the culture vessels. These ciliates proved to be excellent test species and were tested at 5°C and 18°C. All tests only were executed with PO+catalase, except for the 5 day exposure at 5°C, which was also tested with PO alone. As the results of the latter two tests were the same, the average of these two tests is presented. The results are shown in Figure 7.
After only 1 day exposure, the effect is more severe at 18°C compared to 5°C, but after 5 day exposure it is reversed.

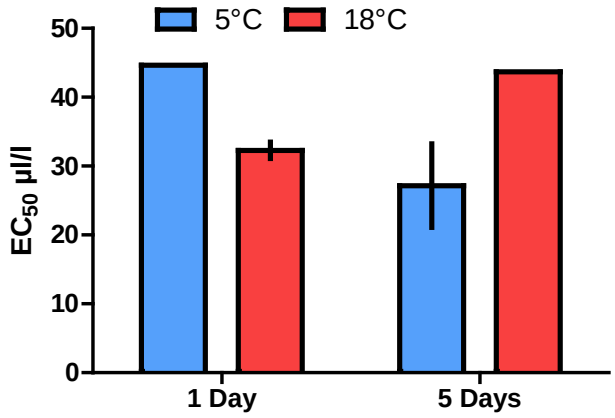

Figure 7 Effect of PO (inclPO+cat) on marine ciliates. Variation between replicated tests indicated with error bar.

As the test was developed during the project, these first tests were executed on 4 different test days, with an incomplete experimental set-up each time (only one temperature and/or exposure period in a test). The test was repeated in a full set-up, to eliminate differences in community sensitivity in time and to incorporate growing experience in analysis. The results confirmed the recovery after 5 days in the 18°C treatment and also showed that at this temperature PO seems to be less effective than

PO+Catalase, whereas at 5°C there is no difference (Figure 8 left). The combined data for PO + catalase are shown in Figure 8 right.

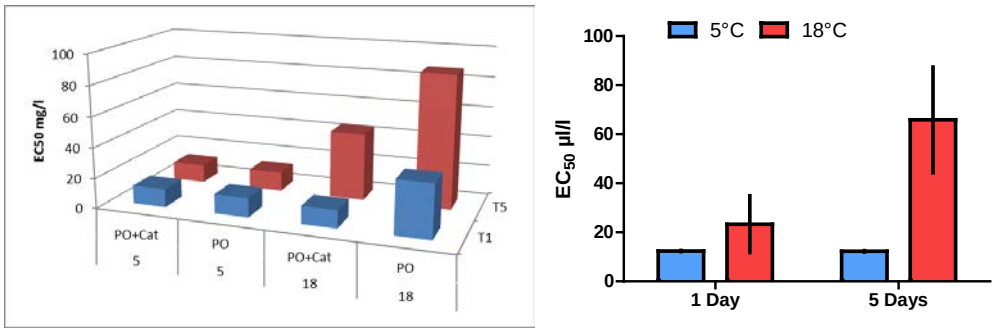

Figure 8 Effect of PO and PO+Cat on cultured ciliates at two test temperatures, measured after 1 day (T1) and 5 days (T5) exposure. Left: final test. Right: combined data for PO+Cat, showing recovery after 5 days.

Additionally, a test with a natural developed community of marine rotifers was conducted at 18°C. In this test, the concentration range chosen was too low. For PO concentrations ranging between 0.2 and 1.2 mg/l the effect size (mortality) ranged from 1 to 25%, without clear dose-response relationships.

Monitoring natural communities

From October on, the natural freshwater community developing at the proposed test location in Den Helder was monitored. Early November, flagellates were dominant in the community. These were tested at 8°C. Even at the lowest concentration tested (12.5 mg/l), the effect was more than 85% (Figure 9). It was, therefore, not possible to calculate EC_{50}-values. After 1 day exposure, the effect of PO+catalase was significantly less than the effect of PO alone (t-test, $p<0.001$).

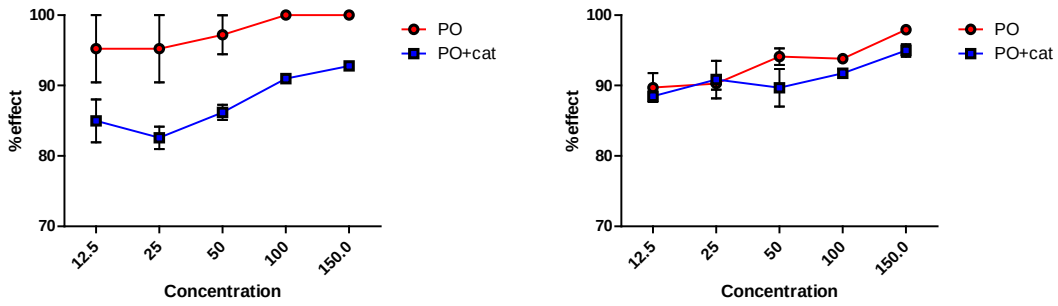

Figure 9 Effect of PO and PO+cat on natural freshwater flagellates, after 1 day (left) and 5 day (right) exposure. Sampled in December

When the test was repeated with lower test concentrations in January, the first test conducted again at 8°C also showed significantly less toxicity for PO without catalase, but a test at 2°C (2 weeks later) showed clearly increased toxicity for the PO treatment, while the toxicity of PO+catalase was slightly less. In these tests, 100% mortality was observed at a concentration of 100 mg/l and 150 mg/l. At 2°C, EC_{50}-values after 5 days were lower than after 1 day, but higher after 4 days.

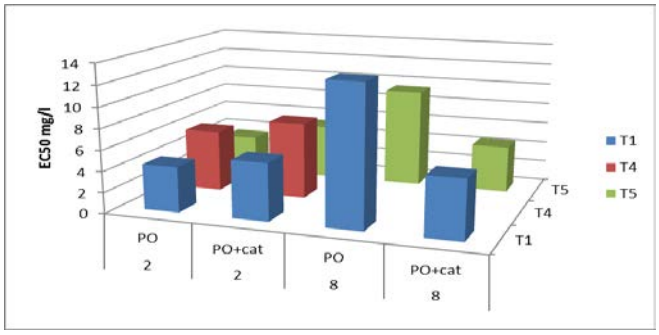

Figure 10 *EC_{50} for natural freshwater flagellates, tested with 2 treatments at 2 and 8°C in January. Analysis at Day 1 (T1), 4 and 5.*

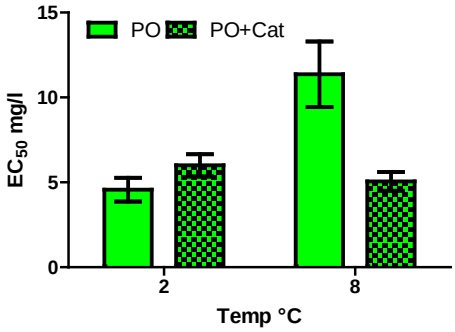

Figure 11 *EC_{50} for natural freshwater flagellates exposed to PO or PO+catalase at two different temperatures (see Figure 10). Measurements at 1, 4 (2°C) and 5 days were averaged.*

5. Discussion

The basic purpose of this research project was to find out whether the prolonged presence , due to a slower degradation, of PAA in ballast water at low temperatures would result in higher toxicity. This effect might be counteracted by slower physiological processes within the target organisms. For this reason, also tests with the standard reference toxicant potassium dichromate (RT=$K_2Cr_2O_7$) were conducted for comparison, as this compound is not biodegraded and is, therefore, expected to be more stable in time. Where possible, also treatment with PO alone was compared with treatment using PO + catalase, as the latter treatment is now proposed for use in BWMS in order to meet discharge standards for H_2O_2, a secondary component of PO. As biodegradation problems at low temperatures were most obvious in freshwater, a comparison was made for the effects in freshwater and sea water.

In general, it seems that toxicity of PO does increase at lower temperatures for marine species, whereas this is less obvious for RT. This effect is not seen in the freshwater tests, or even suggests an opposite direction of the effect. However, the number of successful freshwater tests was very limited.

Interpretation of the effects are impeded by the fact that the effects of PO alone and PO with catalase are not consistent between treatments. Catalase is used to remove hydrogen peroxide from PO and is not expected to have a direct effect on toxicity. As indirect effect a slight increase of toxicity might be observed as the main biocide in PO, peracetic acid, degrades more rapidly in the presence of hydrogen peroxide. Indeed, the toxicity of PO+catalase seems to be slightly higher at higher temperatures, but it disappears at low temperatures, as toxicity of PO alone reaches the same level as PO+catalase.

The most interesting phenomenon is the recovery observed in the test with the marine ciliates. At 5°C recovery was limited, but at 18°C the lowest concentrations fully recovered after 5 days, resulting in a strong increase of the EC_{50}. At 5°C, the effects after 1 day exposure were comparable to the 18°C exposure, but the EC_{50} did not increase over time. This might indicate that prolonged presence of PAA in the system rather prevents recovery, than induces additional toxicity. This hypothesis needs further studies on community level.

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Quality Assurance

IMARES utilises an ISO 9001:2008 certified quality management system (certificate number: 124296-2012-AQ-NLD-RvA). This certificate is valid until 15 December 2015. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V. Furthermore, the chemical laboratory of the Fish Division has NEN-EN-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 1th of April 2017 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation.

Justification

Report number: C088/13
Project number: 430.511103.01

The scientific quality of this report has been peer reviewed by the a colleague scientist and the head of the department of IMARES.

Approved: S. Glorius
Researcher

Signature: 

Date: 27 May 2013

Approved: MSc M. de Wit
Head of department Experimental Ecology

Signature: 

Date: 27 May 2013

Appendix A. General description standard tests

Acute mortality test with the rotifers *Brachionus calyciflorus* and *B. plicatilis*

The tests are performed in a multiwell testplate using freshly hatched larvae of the rotifer *Brachionus calyciflorus* (freshwater) or *B. plicatilis* (salt water), obtained as dried cysts from Microlan B.V., The Netherlands, conform the Standard Operational Procedure for ROTOXKIT (MicroBioTests Inc.). Cysts are incubated in artificial water 28-30h prior to the start of the tests, at 25°C with continuous illumination. After 28-30 hours, most cysts are hatched. Approx. 50 larvae are transferred to a rinsing well containing test medium. From the rinsing well, 5 larvae are transferred to the six wells per concentration/dilution for the test. The well plate is incubated at 25°C in the dark. After 24 hours living and dead larvae in the test wells are counted. Rotifers are considered dead if they do not exhibit any internal or external movement in 5 seconds of observation.

Algae growth inhibition tests with Pseudokirchneriella subcapitata and Phaeodactylum tricornutum

Algal growth inhibition by exposure to a sample is determined by the so-called algal growth inhibition test according to ISO 10253 (2006) procedures using the freshwater algae *Pseudokirchneriella subcapitata* or the marine diatom *Phaeodactylum tricornutum*. After 24, 48 and 72 hours, the growth of algae is analyzed with means of fluorescence readings. The standard test procedure has been adapted by reducing the test volume to 96-well plate levels based on Blaise & Vasseur (2005), Peterson *et al*. (2005) and SCA (2009). Each concentration is replicated eight times and every well contained a total of 0.25 ml in volume. Each test concentration is inoculated with algae from a log-phase growing culture. The start concentration is approx. 10,000 cells/ml, determined microscopically. The 96-well plate is incubated under continuous light at 23±2 resp. 20±2°C and shaken. For each test concentration, a number of wells is not inoculated with algae, in order to allow for correction for context values and water colour. A difference in growth (between the sample and the control) is attributed to the effect of the sample on the organisms.

Appendix B. Summary test results

Table 2 *Summary of test results for marine diatom* P. tricornutum

Testcode	Temp	Time	Test	EC50	95-	95+
Pt120813b	5	144hr	$K_2Cr_2O_7$	5.85		
Pt120813a	5	144hr	$K_2Cr_2O_7$	6.69		
Pt120813	5	144 hr	PO+Cat	1.85	1.78	1.91
Pt120612	5	192hr	$K_2Cr_2O_7$	5.98		
Pt120612a	5	240 hr	PO+Cat	2.89	2.82	2.96
PT120903	10	48 hr	$K_2Cr_2O_7$	15.93	13.87	18.29
PT120612	10	48 hr	$K_2Cr_2O_7$	11.781	8.16	17.01
PT120903	10	72 hr	$K_2Cr_2O_7$	12.04	10.10	13.17
PT120612	10	72 hr	$K_2Cr_2O_7$	12.24	7.15	20.95
PT120903	10	96 hr	$K_2Cr_2O_7$	11.16	10.53	11.82
PT120612	10	96 hr	$K_2Cr_2O_7$	12.18	1.82	81.32
PT120903b	10	48 hr	PO+Cat	43.24	50.14	56.53
PT120903a	10	48 hr	PO+Cat	59.05	50.71	68.77
PT120903b	10	72 hr	PO+Cat	55.84	50.82	61.37
PT120903a	10	72 hr	PO+Cat	58.85	49.56	69.88
PT120903b	10	96 hr	PO+Cat	56.92	47.56	68.13
PT120903a	10	96 hr	PO+Cat	58.94	45.38	76.55
PT120903	20	48 hr	$K_2Cr_2O_7$	16.01	12.86	19.92
PT120822	20	48 hr	$K_2Cr_2O_7$	17.63	14.16	21.96
PT120612	20	48 hr	$K_2Cr_2O_7$	8.86	6.87	11.41
PT120903	20	72 hr	$K_2Cr_2O_7$	13.52	12.26	14.91
PT120822	20	72 hr	$K_2Cr_2O_7$	16.69	14.46	19.28
PT120612	20	72 hr	$K_2Cr_2O_7$	15.07	12.90	17.60
PT120903	20	48 hr	PO+Cat	34.03	30.31	38.21
PT120822	20	48 hr	PO+Cat	42.17	40.63	43.78
PT120903	20	72 hr	PO+Cat	40.13	36.72	43.85
PT120822	20	72 hr	PO+Cat	48.44	-	-

Table 3 *Summary of test results for freshwater algae* Pseudokirchneriella subcapitata

Testcode	Temp	Time	Test	EC50	95-	95+
PS-PC121015	23	72hr	PO+Cat	23.81	21.98	25.80
PS-PO121015	23	72hr	PO	10.34	9.98	10.71
PS-PC121008	23	72hr	PO+Cat	>14.4	-	-
PS-PO121008	23	72hr	PO	9.01	8.29	9.80
PS-RT121008a	23	72hr	K2Cr2O7	1.36	1.30	1.43
PS-RT121008b	23	72hr	K2Cr2O7	1.23	1.12	1.35
PS-RT121008c	23	72hr	K2Cr2O7	1.58	1.51	1.66

Table 4 *Summary of test results for the marine rotifer* Brachionus plicatilis

Code	Temp	Time	Test	EC50	95-	95+	Remark
BP25-RT1	25	24h	K2Cr2O7	329.1	293.3	369.4	
BP25-RT2	25	24h	K2Cr2O7	320.4			
BP10-RT3	10	24h	K2Cr2O7	539.6			
BP25-RT4	25	24h	K2Cr2O7	262.8	260.6	265	
BP05-RT5	5	24h	K2Cr2O7	357.3			wide
BP10-RT6	10	24h	K2Cr2O7	496.2			wide
BP25-RT7	25	24h	K2Cr2O7	342.8	241.4	486.8	
BP10-RT8	10	24h	K2Cr2O7	387.7	355.4	422.9	
BP05-RT9	5	24h	K2Cr2O7	326.4			wide
BP25-RT16	25	24h	K2Cr2O7	317.6	292.5	344.9	
BP05-RT18	5	24h	K2Cr2O7	237.1	236.6	237.5	
BP25-PO10	25	24h	PO	13.49	6.35	28.7	
BP10-PO11	10	24h	PO	7.30	7.30	7.31	
BP05-PO12	5	24h	PO	6.19			
BP25-PO19	25	24h	PO	9.87	9.85	9.89	
BP10-PO20	10	24h	PO	5.03	5.03	5.03	
BP05-PO21	5	24h	PO	5.03	5.03	5.03	
BP25-CA1	25	24h	PC	9.58	9.56	9.59	
BP05-CA3	5	24h	PC	6.12			wide
BP25-CA4	25	24h	PC	8.50	8.48	8.51	
BP10-CA5	10	24h	PC	5.95			
BP05-CA6	5	24h	PC	6.03			
BP25-RT1	25	48h	K2Cr2O7	181.5			wide
BP25-RT2	25	48h	K2Cr2O7	196.0			
BP10-RT3	10	48h	K2Cr2O7	314.4	304.2	324.9	
BP25-RT4	25	48h	K2Cr2O7	204.1	74.92	556.1	
BP05-RT5	5	48h	K2Cr2O7	319.2			wide
BP10-RT6	10	48h	K2Cr2O7	287.5	266.7	310.1	
BP25-RT7	25	48h	K2Cr2O7	342.8	241.4	486.8	
BP10-RT8	10	48h	K2Cr2O7	387.7	355.4	422.9	
BP05-RT9	5	48h	K2Cr2O7	319.6			wide
BP25-RT16	25	48h	K2Cr2O7	317.6	292.5	344.9	
BP25-PO10	25	48h	PO	7.16			wide
BP10-PO11	10	48h	PO	7.30	7.30	7.31	
BP05-PO12	5	48h	PO	6.13			wide
BP25-PO19	25	48h	PO	10.95			
BP10-PO20	10	48h	PO	5.03	5.03	5.03	
BP05-PO21	5	48h	PO	3.94			
BP25-CA1	25	48h	PC	8.92	8.89	8.95	
BP05-CA3	5	48h	PC	4.35			wide
BP25-CA4	25	48h	PC	7.74	7.74	7.74	
BP10-CA5	10	48h	PC	5.38			
BP05-CA6	5	48h	PC	5.38			

Table 5 *Summary of test results for the freshwater rotifer* Brachionus calyciflorus

Testcode	Temp	Time	Test	EC50	95-	95+	Remark
BC25-RT13	25	24hr	K2Cr2O7	20.91	14.12	30.98	
BC10-RT14	10	24hr	K2Cr2O7	>31			
BC05-RT15	5	24hr	K2Cr2O7	>31			
BC25-RT19	25	24hr	K2Cr2O7	23.31	19.17	28.35	
BC10-RT20	10	24hr	K2Cr2O7	>31			
BC25-PO16	25	24hr	PO	<3.6			
BC10-PO17	10	24hr	PO	<3.6			
BC05-PO18	5	24hr	PO	4.58	3.84	5.45	
BC25-PO22	25	24hr	PO	4.33			wide
BC10-PO23	10	24hr	PO	4.49	4.47	4.51	
BC25-CA7	25	24hr	PO+Cat	~4.94			>C5
BC10-CA8	10	24hr	PO+Cat	>31			
BC05-CA9	5	24hr	PO+Cat	5.37	3.15	9.15	>C5
BC25-CA10	25	24hr	PO+Cat	4.35	4.32	4.39	
BC10-CA11	10	24hr	PO+Cat	4.98			>C5
BC25-RT13	25	48hr	K2Cr2O7	>32			
BC10-RT14	10	48hr	K2Cr2O7	27.41	2.42	310.2	
BC05-RT15	5	48hr	K2Cr2O7	28.22	6.81	117	
BC25-RT19	25	48hr	K2Cr2O7	<3.2			
BC10-RT20	10	48hr	K2Cr2O7	36.15	35.62	36.69	>C5
BC25-PO16	25	48hr	PO	<3.6			
BC10-PO17	10	48hr	PO	<3.6			
BC05-PO18	5	48hr	PO	2.89	2.16	3.87	
BC25-PO22	25	48hr	PO	<0.5			
BC10-PO23	10	48hr	PO	2.88	2.76	3.01	
BC25-CA7	25	48hr	PO+Cat	5.90	3.28	10.6	>C5
BC10-CA8	10	48hr	PO+Cat	4.25	3.34	5.41	
BC05-CA9	5	48hr	PO+Cat	3.12	2.00	4.86	
BC25-CA10	25	48hr	PO+Cat	<0.5			
BC10-CA11	10	48hr	PO+Cat	3.07	3.04	3.10	