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Microbial responses to mustard gas dumped in the Baltic Sea

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Abstract

Microbiological studies were carried out on chemical weapon dump sites in the Baltic Sea. The effect of mustard gas hydrolysis products (MGHPs) on marine microbiota and the ability of microorganisms to degrade MGHPs were studied. Many stations at the dump sites demonstrated reduced microbial diversity, and increased growth of species able to use mustard gas hydrolysis products as sole source of carbon. Significant amounts of MGHP-degrading bacteria were revealed in the near-bottom water. The MGHP-degrading microorganisms identified as *Achromobacter* sp., *Pseudomonas* sp., and *Arthrobacter* sp. were isolated. These microorganisms were capable of utilizing the major product of hydrolysis, thiodiglycol, as the sole source of carbon and energy. The bacteria were capable of metabolizing MGHPs at a low temperature. The metabolic pathway for thiodiglycol degradation was proposed. The results suggest the potential for MGHPs biodegradation by naturally occurring populations of near-bottom-water and sediment microorganisms.

Keywords: Bacteria, Baltic Sea, Chemical weapon, Sea dumping, Mustard gas, Degradation, Self purification.

Abbreviations: CWA, chemical warfare agents; MGHPs, mustard gas hydrolysis products; TDG, thiodiglycol; OCS, organochlorine substances; TDGA, thiodiglycolic acid; TGA, thioglycolic acid; HETA, [(2-hydroxyethyl)thio]acetic acid.

1. Introduction

Tens of thousands of tons of captured German chemical munitions have been dumped in the Baltic Sea since World War II. It is estimated that the chemical munitions contained about 13,000 tons of chemical warfare agents (HELCOM, 1994). The dumping of munitions at sea by the allied countries was done in the southern and western Baltic, mainly in the Gotland Basin, the Bornholm Basin, the Little Belt and the Skagerrak Strait (Fig. 1).

In most cases the warfare agents were thrown overboard, either loose (bombs, shells) or in containers, but some ships were also sunk (HELCOM, 1996). During transportation to the dumping areas some munitions were thrown overboard while the ships were en route. Warfare agents are, therefore, assumed to be spread over a considerable area along the transport routes (Schulz-Ohlberg et al., 2001). Chemical munitions have been spread even further by fishing vessels when trawling. Table 1 gives an overview of the quantities of chemical munitions and warfare agents dumped in the Baltic Sea and Skagerrak (Chemical munitions, 1993).

The largest number of chemical agents was dumped in the Bornholm Basin, east of the island of Bornholm. Fishermen still recover bombs, shells and lumps of mustard gas in their nets in this area (HELCOM, 1993).

Over the period 2000-2005 up to 1,010 kg of chemical munitions a year were netted by fishermen east of Bornholm (HELCOM, 2005). The netted munitions were completely corroded and resembled clay-like lumps of mustard gas. The highest number of incidents during the last 20 years was in 1991 with 103 catches amounting to 5,378 kg of chemical warfare agents. The most recent report of recovery of bomb containing mustard gas and tear gas Clark I came from Sweden in 2007 (Åstot et al., 2007).

Most accidents with warfare agents so far have involved mustard gas. Because of the admixture of thickeners, viscous mustard gas is the only warfare agent occurring in large lumps that are mechanically relatively stable and can exist unchanged for decades on the sea bed. All injuries to fishermen have occurred when liquid or solid mustard gas has escaped from rusted munitions and come into contact with the skin. The mustard gas causes severe blistering of the skin and irritates the eyes and throat (Glasby, 1997).

Mustard gas is extremely toxic for all species (Opresko et al., 1998). Because of its high lipid solubility mustard gas rapidly penetrates cells. It is able to alkylate DNA, RNA, and proteins affecting a variety of cell functions (Papirmeister et al., 1991). The products of mustard gas hydrolysis have a wide spectrum of toxic activity on soil microbiota (Medvedeva et al., 2008). They are known to change significantly the number and the specific composition of soil microorganisms (Medvedeva et al., 2006). Contamination of soil with mustard gas causes significant ecological shifts, such as a decrease in the similarity index and the diversity index of microbiota, and a decrease in the dominance index. Microbial succession is accompanied by a decrease

78 in the fermentative activity of soils (Medvedeva et al., 2000).

79 To assess the risk relating to dumped chemical weapons, it is necessary to
80 evaluate the ability of the ecosystem itself (and microbiota as its component) for self-
81 purification of contaminated water and sediments. The ability of microorganisms to
82 carry out mineralization of organic substances, in particular, toxicants of various
83 chemical natures is well known and widely used in practice and scientific research
84 (Oldenhuis et al., 1992). Because of its high degree of toxicity, mustard gas is
85 generally resistant to microbial attack. However, several studies have demonstrated
86 that mustard gas is subject to biodegradation by a limited number of naturally
87 occurring species.

88 Biodegradation of mustard gas by basidiomycetes, *Coriolus versicolor* and
89 *Tyromyces palustris*, has been reported (Wariishi et al., 2002). Bacteria *Micrococcus*
90 *sp.* 6-2 and *Pseudomonas sp.* 8-2 were able to metabolize the products of mustard gas
91 hydrolysis, particularly those that were chlorinated (Medvedeva et al., 2000).
92 Bacterium *Rhodococcus rhodochrous* IQTS8 had the ability to utilize a chemical
93 analog of mustard gas 2-chloroethyl-ethylsulfide as the only carbon and energy source
94 for growth (Kilbane & Jackowski, 1996).

95 More studies are concerned with thiodiglycol (TDG), the major product of
96 mustard gas hydrolysis (Munro et al., 1999). Thiodiglycol is a fairly stable substance
97 with weak toxicity. Nevertheless, it is one of the war gas precursors that must be
98 destroyed, according to the Chemical Weapons Convention. In this connection,
99 biodegradation of mustard gas is closely related to the utilization of TDG by
100 microorganisms. Soil bacteria *Pseudomonas pikettii* SH18 and *Alcaligenes*
101 *xylosoxydans ssp. xylosoxydans* SH42 (Harvey et al., 1993), *Micrococcus sp.* 6-2 and
102 *Pseudomonas sp.* 8-2 (Medvedeva et al., 2000), *Pseudomonas sp.* Y-13 (Medvedeva
103 et al., 2006), *Alcaligenes xylosoxydans* PGH 10 (Garcia-Ruiz et al., 2002),
104 *Alcaligenes xylosoxydans subsp. denitrificans* TD 1 (Ermakova et al., 2002),
105 *Alcaligenes xylosoxydans ssp. xylosoxydans* SH91 (Lee et al., 1996) are capable of
106 using TDG as the sole source of carbon. While degradation of thiodiglycol has been
107 characterized in terrestrial isolates, little is known about marine-degrading
108 microorganisms.

109 The goal of our study was to investigate the effect of warfare agents on the Baltic
110 Sea microbiota. We report in this paper on the isolation, characterization and
111 metabolic activities of the bacteria able to use mustard gas hydrolysis products as the
112 sole source of carbon and energy.

113

114

115 **2. Materials and methods**

116

117 *2.1. Sampling*

118

119 Seawater samples were collected from Baltic Sea chemical weapon dump sites
120 in 1994-2007. The water samples were taken from the Bornholm dump site, Gotland
121 Basin, and Skagerrak Strait. Water samples (100 ml) were collected from the whole
122 water column using sterile bathometers. Near-bottom water (100 ml) was taken from
123 Niemistö corers and put into sterile plastic flasks.

124 125 *2.2. Enumeration of microorganisms*

126
127 The number of heterotrophic microorganisms and content of heterotrophic
128 microorganisms able to degrade mustard gas hydrolysis products (MGHPs) were
129 analyzed immediately after sampling. The total number of microorganisms was
130 determined after conservation with formalin using the standard technique (Tsyban,
131 1980). The number of heterotrophic microorganisms was estimated using inoculation
132 on nutrient agar (1:10) (Kuznetsov & Dubinina, 1989). The incubation time was 3-5
133 days. The content of heterotrophic microorganisms able to degrade MGHPs was
134 determined by inoculation on selective agar with water samples. The incubation time
135 was 7 - 10 days. The selective agar had the following composition: $(\text{NH}_4)_2\text{SO}_4$ 4.0 g/l,
136 KH_2PO_4 1.5 g/l, K_2HPO_4 1.5 g/l, MgSO_4 0.2 g/l, NaCl 5.0 g/l and agar 20.0 g/l.
137 Mustard gas hydrolysis products (16 mg/l of organochlorine substances (OCS) and
138 0.35 mg/l of thiodiglycol) were added to this selective medium as the sole source of
139 carbon.

140 141 *2.3. Isolation of microorganisms, media, and culture conditions*

142
143 Samples from the flasks were streaked onto plates containing mineral salts
144 medium (4.0 g/l $(\text{NH}_4)_2\text{SO}_4$, 1.5 g/l KH_2PO_4 , 1.5 g/l K_2HPO_4 , 0.2 g/l MgSO_4 , 5.0 g/l
145 NaCl) solidified with 1.5% agar. Mustard gas hydrolysis products, including 25 mg/l
146 of organochlorine substances (OCS), were used as the sole source of carbon. The
147 plates were incubated at 20 °C for up to 3 weeks. A rapidly growing, visually distinct
148 colony and a separate, morphologically unique isolate were selected for further
149 analysis and purified by repeated plating.

150 The selected strains of microorganisms were cultivated by three methods:

151 (1) By inoculation into a solid medium with the following composition (g/l):
152 $(\text{NH}_4)_2\text{SO}_4$ 4.0, KH_2PO_4 1.5, K_2HPO_4 1.5, MgSO_4 0.2, NaCl 5.0, thiodiglycol 2.0,
153 CaCO_3 2.5, agar 20.0. The control parameters included the occurrence of growth and
154 dissolution of CaCO_3 , which gives evidence of the formation of acidic products of
155 thiodiglycol degradation;

156 (2) Cultivation under static conditions: in 20-ml test tubes filled with 17 ml of
157 seawater containing MGHPs (32 mg OCS/l and 0.96 g/l TDG), pH 7.8 ± 0.2 . The
158 MGHPs solutions, pre-sterilized separately by filtering through membrane filters with
159 a pore diameter of 0.22 μm , were aseptically introduced into the medium, and
160 simultaneously the test tubes were inoculated with the bacterial culture under

161 scrutiny.

162 (3) Cultivation under aeration: in 250-ml Erlenmeyer flasks on a rotary shaker
163 CERTOMAT BS-1 (B.Braun Biotech Int. GmbH, Germany) at 240 rpm on a medium
164 consisting of (g/l): $(\text{NH}_4)_2\text{SO}_4$, 6.0; KH_2PO_4 , 5.0; K_2HPO_4 , 5.0; MgSO_4 , 0.2; and
165 CaCO_3 , 10.0, containing MGHPs (20 - 750 mg /l OCS; 6 – 22.5 g/l TDG). Control
166 parameters included the biomass growth, the change in pH of the culture broth, the
167 presence of TDG, and the content of organochlorine substances.

168 The biomass growth was routinely monitored by measuring D_{540} with a Genesys
169 10S spectrophotometer (Thermo Electron Corp., USA).

170 Considering that chemical hydrolysis of organochlorine substances can proceed
171 in water medium, the studies of microbial degradation of MGHPs were carried out in
172 comparison with the control probe (with the process performed under the same
173 conditions but without microorganisms).

174

175 2.4. DNA extraction from bacterial cultures

176

177 DNA was extracted from the culture samples using incubation with 0.75 ml of
178 1% (w/v) potassium ethyl xanthogenate containing 1% SDS (w/v), 20 mM EDTA,
179 100 mM TRIS and 800 mM $\text{CH}_3\text{COONH}_4$, pH 7.4 (70 °C, 1 h), followed by vortexing
180 (10 s), a second incubation (4 °C, 30 min) and centrifuging (17 530 x g, 4 °C, 10 min).
181 DNA was precipitated using 0.75 ml of 2-propanol (22-24 °C, 10 min), pelleted (12
182 000 x g, 22-24 °C, 10 min), washed twice with 200 μl of 70% (v/v) ethanol, air dried
183 (20-24 °C) and dissolved in 50-200 μl of 10 mM TRIS, 1 mM EDTA, pH 8.0 (TE
184 buffer). The DNA isolates were further purified using Montage cartridges (Millipore,
185 Billerica, CA, USA) and stored at -20°C until amplification with polymerase chain
186 reaction (PCR).

187

188 2.5. DNA amplification

189

190 The primers were from Oligomer (Helsinki, Finland). Partial 16S rRNA genes
191 were amplified from the culture samples using a forward primer 27F (5'-
192 AGAGTTTGATCCTGGCTCAG-3'; general bacterial primer; Lane 1991) and a
193 reverse primer 1525R (5'-AAGGAGGTGATCCAGCC-3'). PCR was performed in 20
194 μl scale with 1 μl of template DNA, 0.4 μM of forward and reverse primers, 200 μM
195 of each deoxynucleoside triphosphate (dNTP, Sigma-Aldrich), 1 U of MasterTaq
196 polymerase (Eppendorf, Hamburg, Germany), 1 x MasterTaq polymerase buffer
197 containing 1.5 mM $\text{Mg}(\text{OAc})_2$, 0.5 x Taq Master Enhancer (Eppendorf), and 3 g l⁻¹
198 BSA. A Thermo PCR Sprint (Milford, MA, USA) thermal cycler was used. The
199 initial step in the temperature programme was 70 °C (5 min) and 94 °C (5 min). DNA
200 polymerase was added during the initial 70 °C period (hot start). The following
201 denaturing, annealing and extension programmes were used: 9 cycles of [94 °C (45 s),

202 57 °C (45 s), 68 °C (2 min)], 24 cycles of [94 °C (45 s), 54 °C (45 s), 68 °C (2 min)]
203 and 68 °C (7 min). The formation of PCR products (approx. 1,500 bp) was checked
204 using agarose gel electrophoresis. The PCR products were stored at -20 °C. Products
205 used in cloning were stored for a maximum of 24 h.

206

207 2.6. Cloning and sequencing

208

209 The cloning of amplified DNA was performed with a Qiagen PCR cloning kit
210 (pDrive plasmid vector) at the Sequencing Laboratory of the Institute of
211 Biotechnology, University of Helsinki. After a 24-h culturing bacterial pellets were
212 lysed and plasmid DNA purified using 2-propanol precipitation and centrifuging, and
213 stored in TE (-20 °C). The plasmids were further amplified with primers UP and RP
214 (5'-TAACGCCAGGGTTTTCCAGTCACGA-3' and 5'-
215 GTGAGCGGATAACAATTTTCACACAGG-3'), amplifying the insert from the
216 plasmid. The products were isolated, subjected to sequencing reactions (two
217 directions; primers t7 and sp6; 5'-TAATACGACTCACTATAGGG-3' and 5'-
218 ATTTAGGTGACACTATAGAA-3') and sequenced (ABI3730 XL 48-capillary
219 automated sequencer). Sequences without overlapping or unclear bases were
220 subjected to preliminary analysis using the Basic Local Alignment Search Tool
221 (BLAST) programme (National Center for Biotechnology Information, Bethesda,
222 MD, USA).

223

224 2.7. Chemicals

225

226 A model mixture of mustard gas hydrolysis products was prepared by heating an
227 aqueous mixture of 0.65 M TDG (ICN 103039 RT, 98% purity) and 0.65 M HCl at 90
228 °C for 8 h. The resulting MGHPs mixture contained $2\,500 \pm 300$ mg/l OCS and $(0.57$
229 $\pm 0.01)$ M TDG. Thiodiglycolic acid (TDGA), thioglycolic acid (TGA) and β -
230 mercaptoethanol were obtained from Merck KgaA, Germany. All the chemicals were
231 of the highest purity commercially available. [(2-hydroxyethyl)thio]acetic acid
232 (HETA) was synthesized and purified according to Black et al. (1993).

233

234 2.8. Analytical methods

235

236 The quantitative determination of OCS was performed spectrophotometrically
237 (Franke, 1967). Thiodiglycol, thiodiglycolic acid, [(2-hydroxyethyl)thio]acetic acid
238 and thiodiglycol sulphoxide content were determined by reversed-phase HPLC on an
239 HP 1090 Hewlett-Packard chromatograph under the following conditions:
240 temperature 25 °C, Luna (Phenomenex) column; flow rate 1.5 ml/min; detection at
241 215 nm; eluent 5% acetonitrile and 0.1% TFA (trifluoroacetic acid); sample volume
242 20 μ l.

243 Hydrolysis of MGHPs was also determined by measuring the release of chloride
244 ions with a model 96-17 B combination electrode (Orion Research Inc., USA). The
245 electrode was calibrated by adding known concentrations of sodium chloride
246 standards to the mineral salts medium used in the experiments. Uninoculated flasks
247 were run in each experiment as controls.

248 Thioglycolic acid was detected by using a Hewlett-Packard 1090 HPLC liquid
249 chromatograph with a diode array detector (USA) equipped with a Vydac column
250 (USA) 218TP54 (RP-18, 4.6×250 mm, particle size 5 µm) under isocratic conditions
251 with 20 mM tetrabutyl ammonium (TBA) buffered with 20 mM Na phosphate
252 (pH=6.0), containing 15% (v/v) acetonitrile at 50 °C. The flow rate was 1.5 ml/min
253 and detection of the compounds was at 210 nm, 250 nm, and 265 nm. The injection
254 volume was 10 µl and the analysis lasted 10 min.

255 Detection of β-mercaptoethanol after its modification by Elman (1959) was
256 carried out on a Hewlett-Packard 1090 HPLC liquid chromatograph with UV Monitor
257 Model 1740 BIO-RAD (USA) using Waters C-18 column, (Δ-Pack, 3.9×150 mm,
258 particle size 5 µm) gradient mode (1.5 ml/min). The eluents were: A, 0.1% TFA and
259 B, 100% acetonitrile. For the analysis, the following linear gradient was used: from
260 20% B (0 min), 37% B (8.5 min) at 20 °C. The total analysis time was 12 min. The
261 compounds were detected at 313 nm and 338 nm.

262 263 2.9. Statistical analyses

264
265 All statistical analyses were performed with Statistica software (version 6;
266 Statsoft). The differences were considered significant at $P < 0.05$.

267 268 269 3. Results and discussion

270 271 3.1. Effect of MGHPs on marine microbiota

272
273 Over the last few decades microbiological studies have been carried out at the
274 chemical munitions dump sites in the Baltic Sea. In 1994-2000, we monitored the
275 ecological situation at the dump sites under the Marine Ecological Patrol Program.
276 The analysis of the near-bottom water samples taken while we were sailing in the
277 Skagerrak Strait, as well as near Liepaja and at the Bornholm dump site yielded $0.5 \cdot$
278 10^6 to $7.1 \cdot 10^6$ cells/ml for the total amount of microbiota, 0.4 to 6.5 mg/l for
279 bacterial biomass, and $0.2 \cdot 10^4$ - $4.9 \cdot 10^4$ cells/ml for the amount of heterotrophic
280 microorganisms (Table 2).

281 At many stations significant amounts of bacterial cultures, able to grow in the
282 medium containing mustard gas hydrolysis products (both chlorinated and non-
283 chlorinated), were observed in the near-bottom water. However, there was no
284 significant growth of these microorganisms following inoculation of water samples to

285 the same agar medium without MGHPs. It was therefore concluded that the source of
286 carbon for these bacteria were MGHPs. As Table 2 shows, at some stations they
287 account for 20-98% of the total heterotrophic microbiota. No MGHP-degrading
288 microorganisms were found in surface water layers and horizons 1 m above the sea
289 bed. The formation of an enrichment culture of microorganisms at some of the
290 observation stations can be due to the appearance in the environment of a new
291 compound, mustard gas, acting as a selective agent. The largest number of
292 "abnormal" sites was observed in the Bornholm Basin. MGHP-degrading
293 microorganisms appeared at about 40% of stations in this area (Medvedeva et al.,
294 1998).

295 In 2007, the microbiological studies were carried out in the Bornholm dump site
296 area in the framework of the EC-FP6 project "MERCW" (Modelling of
297 Environmental Risks Related to Sea-dumped Chemical Weapons). The studies
298 focused on the primary dumping area located in the southern part of the Bornholm
299 Basin, east of the island of Bornholm, in water depths ranging from 70 to over 95 m
300 (Fig. 2). Four shipwrecks were identified in this area in 1994-2000 (Paka and
301 Spiridonov, 2001). The wrecks were extensively damaged. One of the wrecks was
302 covered by trawling nets, which were suspended in the water column above the
303 wrecks by intact floats (Fig. 3A). Partly corroded artillery missiles were observed on
304 the deck of the other wreck (Fig. 3B). The microbiological survey was undertaken in
305 two research areas (Fig. 2): the first research area was in the vicinity of the
306 shipwrecks and the second in the zone marked by the detection of chemical warfare
307 agents on previous research trips (Paka, 2004).

308 Near-bottom water contained MGHP-degrading microorganisms that were up to
309 58% of the total number of heterotrophic microorganisms at more than a half of the
310 stations (Fig. 4). Most of such "abnormal" points with a high concentration of MGHP-
311 degrading microorganisms were detected in the first research area near the sunken
312 ships. 7 "abnormal" points were found in the second research area, where CWA had
313 been detected previously.

314 The "abnormal" sites of mustard gas possibly leaking into the environment
315 differed from other stations in the species diversity of microorganisms (Table 3).

316 The microbiota of the near-bottom water out of the dump site (30 km) was
317 represented by microorganisms predominantly of the genera *Pseudomonas*, *Bacillus*,
318 *Sphingomonas*, *Flavobacterium*, *Micrococcus*, *Alcaligenes*, *Achromobacter*, with
319 *Pseudomonas* and *Bacillus* genera dominating (the latter accounted for up to 25% of
320 the total number of heterotrophs).

321 The primary dump site showed a reduced diversity of bacteria. At many stations
322 the dominance of a minimal number of *Pseudomonas*, *Bacillus*, *Arthrobacter*, and
323 *Achromobacter* species in near-bottom water was observed. The species diversity of
324 the microbial population at the dump site was reduced compared with other sites,
325 most probably because of an increase in the number of MGHP-degrading

326 microorganisms. The reduction in the species diversity of bacterioplankton suggests a
327 decrease in the stability of the biosystem as a whole.

328

329 3.2. Isolation and characterization of MGHP-degrading microorganisms

330

331 During the period 1994-2007, over 200 MGHP-degrading bacteria were obtained
332 from the near-bottom water samples taken at the Gotland Basin, Skagerrak Strait, and
333 Bornholm dump site. Three of the most active strains capable of utilizing mustard gas
334 hydrolysis products, 75-1, 23-3 and 93-2, were obtained from the Bornholm dump
335 site.

336 All three isolated bacterial strains were aerobic, oxidase and catalase-positive, –
337 two were gram-negative, and one was gram-positive. The gram-positive strain 23-3,
338 established by phenotypic assays, underwent a rod-coccus morphological change.
339 Strain 75-1 was the gram-negative, nonmotile rod of ~ 0.5 by 1.5 to 2.0 µm. Strain
340 93-2 was the gram-negative, rod-shaped motile organism about 1.0 by 1.5 µm.
341 Motility was imparted by a single polar flagellum. All three strains grew between 0 °C
342 and 30 °C, with the optimal growth temperature around 25 °C.

343 The 16S rRNA gene sequences (420-470 bp) showed the greatest similarities
344 (99%) with strain 75-1 and were related to those of the *Achromobacter* genera. Strain
345 23-3 was most closely related to bacteria from the genus *Arthrobacter* (with sequence
346 similarities ranging from 98 to 99%). The results indicated that strain 93-2 was
347 phylogenetically closest to *Pseudomonas*.

348 The genera previously implicated in the degradation of mustard gas hydrolysis
349 products include the genera *Alcaligenes* (Lee et al., 1996, Mulbry et al., 1998),
350 *Pseudomonas* (Harvey et al., 1993; Medvedeva et al., 2007), and *Micrococcus*
351 (Medvedeva et al., 2000). These bacteria have been isolated from contaminated
352 terrestrial environments or from soil enrichment cultures by using mustard gas
353 hydrolysis products. The present results indicate there are also marine MGHP-
354 degrading bacteria of genera *Arthrobacter*, *Achromobacter*, and *Pseudomonas*.
355 Although *Achromobacter* (also known as *Alcaligenes*) and *Pseudomonas* strains have
356 been characterized as microorganisms capable of metabolizing the products of
357 mustard gas hydrolysis, MGHP-degrading *Arthrobacter* has been found for the first
358 time.

359

360 3.3. MGHP-degradation range

361

362 Comparative cultivation of degrader microorganisms was carried out (Table 4).
363 The highest MGHPs-dechlorinating capability was exhibited by *Achromobacter sp.*
364 75-1. The maximum dechlorination rates were 26 mg OCS/g biomass/day under static
365 conditions, and under aeration, 37 mg OCS/g biomass/day. All strains had shown
366 lower dechlorination rates under static conditions than under aeration. The process of

367 dechlorination was accompanied by evolution of the stoichiometric amount of mineral
368 chlorine in the medium.

369 The degrader microorganisms also grew more actively under aeration. For
370 example, the biomass gain for *Achromobacter sp.* 75-1 under aeration was 8 times
371 that under static conditions. Figure 5 shows the dynamics of growth of
372 *Achromobacter sp.* 75-1 in a medium supplemented with MGHPs as the sole carbon
373 and energy source. The growth of microorganisms correlates with the decrease in
374 thiodiglycol content. The complete utilization of MGHPs (60 mg OCS /l and 0.18%
375 TDG) is observed after 6 days.

376 Figure 6 shows the effect of different concentrations of mustard gas hydrolysis
377 products on the bacterial growth under aeration. The growth of strain 75-1 (Fig. 6a)
378 was proportional to the concentration of OCS up to 360 mg/l in the medium.
379 However, concentrations above 360 mg/l induced a lag phase in bacterial growth.
380 This effect was more pronounced at OCS concentrations above 500 mg/l.
381 Concentrations equal to or greater than 750 mg/l totally suppressed the growth.

382 Increasing concentrations of OCS adversely affected the biomass growth of both
383 strain 93-2 (Fig. 6b) and strain 23-3 (Fig. 6c). The concentration of OCS also affected
384 the lag phase before the beginning of TDG degradation by *Arthrobacter sp.* 23-3 and
385 *Pseudomonas sp.* 93-2. The higher the concentration of OCS, the longer the lag phase
386 lasted before the start of degradation (data not shown). The lag period before TDG
387 degradation at high OCS concentrations was shown to be a lag phase in cell growth.
388 Concentrations of OCS equal to or greater than 240 mg/l totally suppressed the
389 growth of *Arthrobacter sp.* 23-3 and *Pseudomonas sp.* 93-2.

390 The highest specific growth rate (μ_{\max}) of 0.89 day^{-1} was at 360 mg of OCS/l for
391 strain *Achromobacter sp.* 75-1 (Fig. 7).

392 The highest growth rates for strains *Arthrobacter sp.* 23-3 and *Pseudomonas sp.*
393 93-2 were $0.76 - 0.73 \text{ day}^{-1}$ at 120 and 60 mg of OCS/l, respectively. The growth rates
394 decreased in proportion to an increasing OCS concentration, and the lowest growth
395 rate of 0.1 h^{-1} was observed at 730 mg of OCS/l (strain 75-1) and 260 mg of OCS/l
396 (strains 93-2 and 23-3). The growth of strains 75-1, 93-2 and 23-3 is characterized by
397 the peak of the maximum possible growth rate. Such growth is typical of the growth
398 of microorganisms on a toxic substrate (Karasevitch, 1982).

399 The concentration of MGHPs produces a considerable effect on the process of
400 the dechlorination of OCS, as well as on the growth of bacteria. The ultimate
401 concentration of OCS (360 mg per litre) for the growth of *Achromobacter sp.* 75-1
402 corresponds to the highest rate of dechlorination. The increase in the concentration of
403 OCS above this level leads to the sharp decrease in the rate of dechlorination as well
404 as in the specific growth rate.

405 The growth rate of *Arthrobacter sp.* 23-3 and *Pseudomonas sp.* 93-2 reaches its
406 maximum value at an OCS concentration of 60 - 120 mg/l, but the most rapid
407 dechlorination takes place at higher concentrations of the toxicant (240 mg/l). At this
408 concentration the growth of bacteria is practically absent, and the whole substrate is

409 consumed for energy of maintenance. The low maximum growth rate, long generation
410 time and considerable outlays for the maintenance of both cultures are characteristic
411 of the growth of microorganisms on toxic substrates (Pirt, 1975).

412 413 3.4. Effect of environmental conditions on biodegradation

414
415 The pH range for good growth of degrader bacteria under aeration is 7.0 to 8.0
416 (Fig. 8). There was little difference in growth in cultures with an initial pH between
417 6.0 and 8.0 (averaging about 20%). The growth of *Achromobacter sp.* 75-11 and
418 *Arthrobacter sp.* 23-3 was slightly more rapid at a neutral pH. There was a gradual
419 decrease in the growth rate of *Arthrobacter sp.* as the initial culture pH was increased
420 from 8 to 9. *Pseudomonas sp.* 93-2 grew better in a slightly alkaline rather than acidic
421 condition. The maximum growth rate of strain 93-2 was observed at pH 8.

422 We found that the fastest growth of degrader-microorganisms occurred at 20 and
423 25 °C (Table 5). The growth rate at 20°C ($\mu_{\max} = 0.23 - 0.31$ g/g/d) was about 3-5
424 times more rapid than the growth rate at 5 °C. Cultures also took longer to degrade
425 TDG at 5 °C (Fig. 9, Fig. 10, Fig. 11). The time required for thiodiglycol degradation
426 was 30-45 days at 20 and 25 °C, but 180-210 days were required to degrade TDG at 5
427 °C. The temperature had little effect on the biomass yield (Y) of the degrader-
428 microorganisms (Table 5). However, the data indicated that the optimum temperature
429 for biomass yield was 20–25 °C.

430 The ability of microorganisms to degrade thiodiglycol at a low temperature is
431 particularly important in cold Baltic Sea conditions.

432 433 3.5. Metabolic pathways

434
435 The metabolic pathway for the degradation of MGHPs by marine bacteria was
436 studied. The intermediate products from the growth of strains 93-2, 23-3, and 75-1 in
437 the medium containing thiodiglycol as a source of carbon were analyzed to elucidate
438 the possible pathways of degradation. The disappearance of TDG was accompanied
439 by an accumulation of acids. HPLC analysis revealed thiodiglycolic acid (TDGA)
440 and [(2-hydroxyethyl)thio]acetic acid (HETA) in the culture supernatants of all the
441 strains (Fig. 12). Thiodiglycol sulphoxide was not detected.

442 The appearance of acid products suggests that biodegradation of TDG begins
443 with oxidation of its primary alcoholic groups. Earlier, oxidation of the thiodiglycol
444 molecule to TDGA by alcohol dehydrogenase was reported (Lee et al., 2000,
445 Ermakova et al., 2002). Incubation of all strains for more than 7 days led to a decrease
446 in HETA and TDGA concentrations to an undetectable level.

447 TGA and acetate were detected in the culture supernatant from the growth of all
448 bacteria on the TDGA-containing medium, which suggests C-S bond cleavage in
449 TDGA to yield these products. β -mercaptoethanol was detected as an intermediate
450 product of TDG transformation in only two strains: *Pseudomonas sp.* 93-2 and

451 *Achromobacter sp.* 75-1.

452 These results indicate that the major pathway for TDG degradation by all the
453 strains studied is the oxidation of its primary alcoholic groups to HETA, TDGA and
454 TGA with the formation of acetate upon further transformation. This pathway for
455 TDG degradation is similar to that reported earlier for *Alcaligenes* and *Pseudomonas*
456 (Lee et al., 2000; Garcia-Ruiz et al., 2002, Medvedeva et al., 2006). However, the
457 formation of β -mercaptoethanol was detected for *Pseudomonas sp.* 93-2 and
458 *Achromobacter sp.* 75-1. Previously, this intermediate product has also been detected
459 among the products of TDG transformation by soil bacterium *Pseudomonas sp.*
460 (Medvedeva et al., 2007). Thus, the results suggest two possible pathways of the TDG
461 biodegradation, one of which yields HETA, TDGA and TGA, and the other, β -
462 mercaptoethanol, which is further transformed to TGA (Fig. 13).

463

464 4. Conclusions

465

466 Microbiological analysis of near-bottom water samples near the sea bed of the
467 dump sites for chemical weapons in the Baltic Sea revealed high levels of
468 microorganisms able to use mustard gas hydrolysis products as source of carbon
469 especially at the Bornholm dump site, as well as in the Gotland Deep, and at the
470 Skagerrak dump site. The "abnormal" sites showed reduced diversity of microbiota.
471 High numbers of MGHP-degrading microorganisms in near-bottom water indicate a
472 possible leakage of CW agents into the environment and contamination of water and
473 sediments with mustard gas and products of its hydrolysis in the research areas.

474 Bacterial cultures isolated from near-bottom water were able, with differing
475 biodegradation rates, to dechlorinate and utilize mustard gas hydrolysis products as
476 the source of carbon. The *Arthrobacter*, *Achromobacter* and *Pseudomonas* genera
477 cultures were the most active. The main features of *Arthrobacter sp.* 23-3,
478 *Achromobacter sp.* 75-1, and *Pseudomonas sp.* 93-2 are the tolerance to mustard gas
479 hydrolysis products, and the ability to degrade a high concentration of OCS. They can
480 use thiodiglycol as the sole source of carbon and energy. The isolated bacteria can
481 completely dechlorinate OCS at up to 750 mg/l in 4-9 days. But the complete
482 utilization of the products of hydrolysis (mainly thiodiglycol) is observed at lower
483 concentrations of OCS in the medium (up to 360 mg). The major pathway of TDG
484 degradation was oxidation to HETA, TDGA and TGA plus eventual formation of
485 acetate. The degradation of TDG by *Pseudomonas sp.* and *Achromobacter sp.* 75-1
486 can also occur via C-S bond cleavage yielding β mercaptoethanol. The
487 microorganisms cannot only detoxify the contaminants, but also utilize them
488 completely. The rate of biodegradation of mustard gas hydrolysis was temperature-
489 dependent but also occurred at low temperatures.

490 The ability of microorganisms to carry out the mineralization of thiodiglycol at a
491 low temperature is important for the marine environment, particularly for the dump
492 sites, where water temperatures rarely exceed 7°C. The results suggest the potential

493 for self-purification of contaminated water and sediments by natural populations of
494 microorganisms.

495

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497

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505

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619 **Figure legends**

620

621 Fig. 1 - Dump site locations map: (A) the Bornholm site; (B) the Gotland Deep site;
622 (C) the Skagerrack site

623

624 Fig. 2 - Location of the chemical munitions dump site at the Bornholm Basin. The
625 circle marks the primary dump site area; the rectangle marks the larger dump site
626 boundary. 1: the first research area, 2: the second research area

627

628 Fig. 3 – Images of the shipwrecks at the Bornholm dump site (after Paka and
629 Spridonov, 2001). A: wreck covered with fishing nets. B: missile found on the deck of
630 the wreck

631

632 Fig. 4 - Concentration of MGHP-degrading microorganisms (mean \pm SD) present at
633 the primary dump site within the first research area (1), within the second research
634 area (2), and out of the primary dump site (3)

635

636 Fig. 5 - Growth and utilization of TDG by *Achromobacter sp.* 75-1. The data are
637 means \pm SD of three independent measurements.

638

639 Fig. 6 - Effect of MGHPs concentrations on the growth of *Achromobacter sp.* 75-1
640 (a), *Arthrobacter sp.* 23-3 (b), and *Pseudomonas sp.* 93-2 (c).

641

642 Fig. 7 - Effect of OCS on dechlorination rate (A) and growth rate (B) of
643 *Achromobacter sp.* 70-11, *Arthrobacter sp.* 41-3 and *Pseudomonas sp.* 3-1. The data
644 are means \pm SD of three independent measurements.

645

646 Fig. 8 - Effect of pH on growth of *Achromobacter sp.* 75-1, *Arthrobacter sp.* 23-3 and
647 *Pseudomonas sp.* 93-2 (25°C). The data are means \pm SD (n=3).

648

649 Fig. 9 - Effect of temperature on degradation of TDG by *Achromobacter sp.* 75-1
650 under static conditions: (a) TDG concentration; (b) biomass growth (OD₅₄₀). The
651 symbols indicate the means of triplicate experiments. Vertical bars indicate standard
652 deviations.

653

654 Fig. 10 - Effect of temperature on degradation of TDG by *Arthrobacter sp.* 23-3 under
655 static conditions: (a) TDG concentration; (b) biomass growth (OD₅₄₀). The symbols
656 indicate the means of triplicate experiments. Vertical bars indicate standard
657 deviations.

658

659 Fig. 11 - Effect of temperature on degradation of TDG by *Pseudomonas sp.* 93-2
660 under static conditions: (a) TDG concentration; (b) biomass growth (OD₅₄₀). The

661 symbols indicate the means of triplicate experiments. Vertical bars indicate standard
662 deviations.

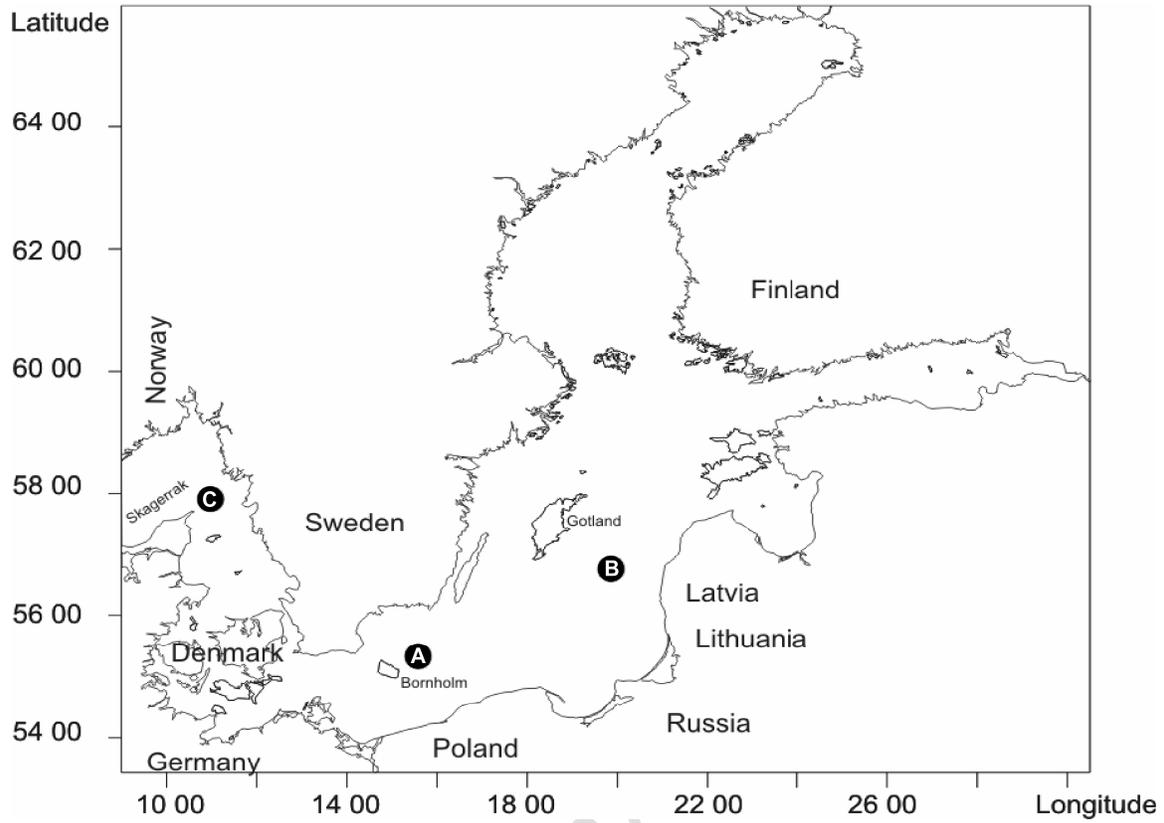
663

664 Fig 12 - HPLC chromatogram of *Arthrobacter sp.* 23-3 supernatant: (1) at initiation of
665 the incubation in TDG-containing media; (2) after 72 hours of incubation

666

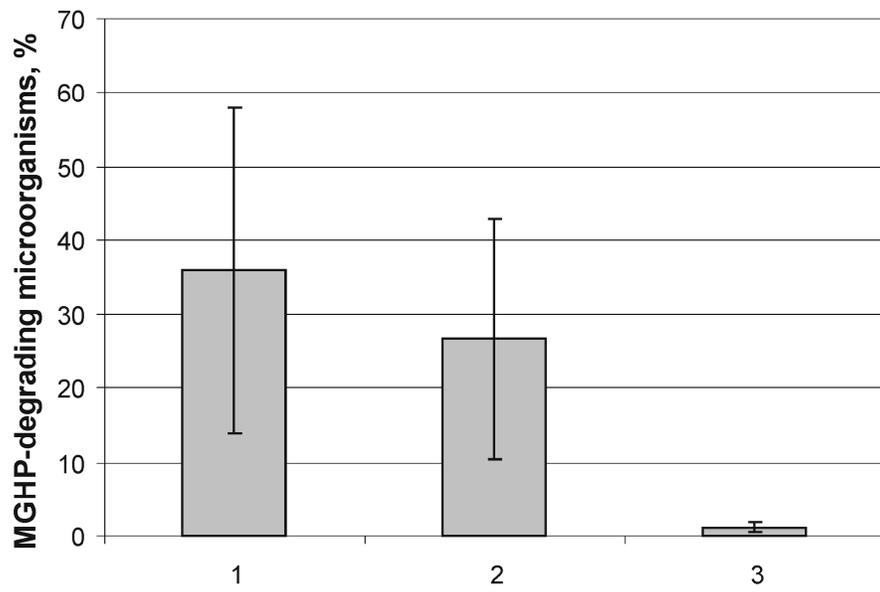
667 Fig. 13 - Metabolic pathways for TDG utilization by bacteria isolated from the Baltic
668 Sea

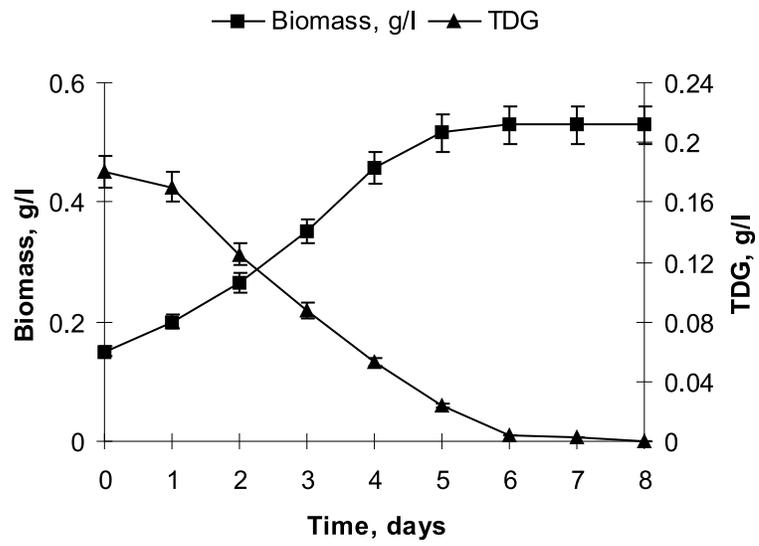
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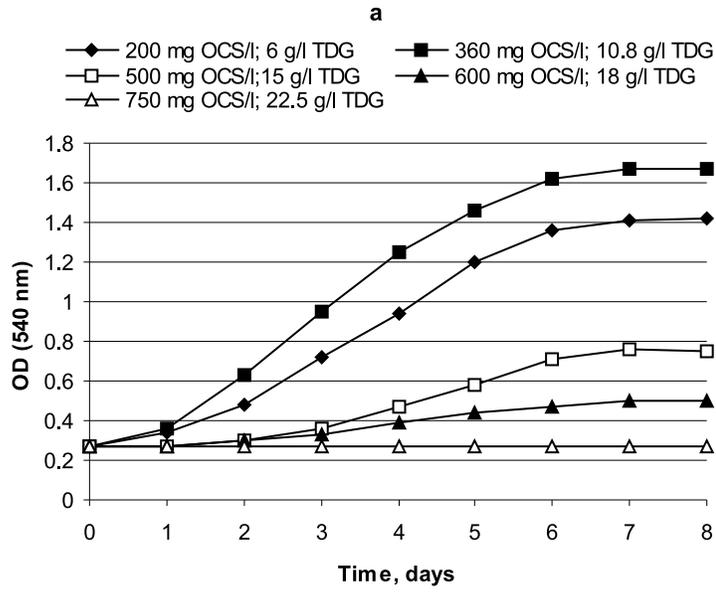


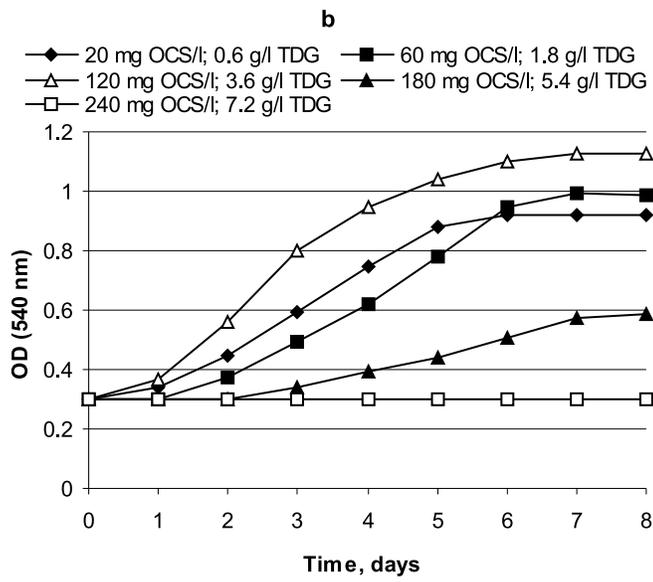


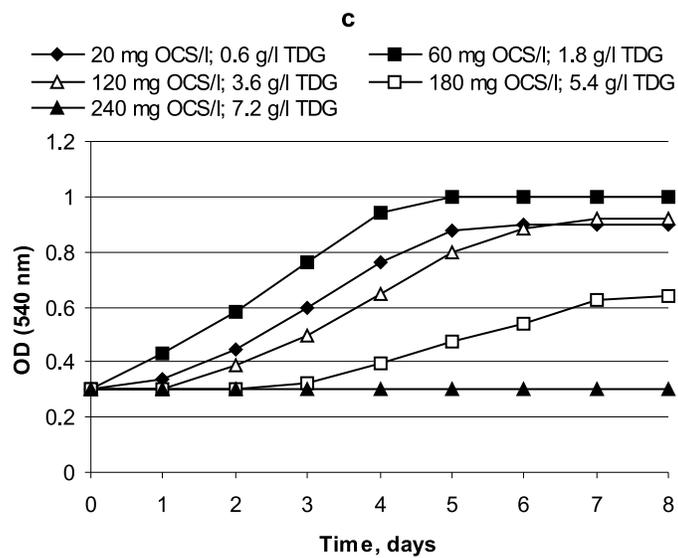
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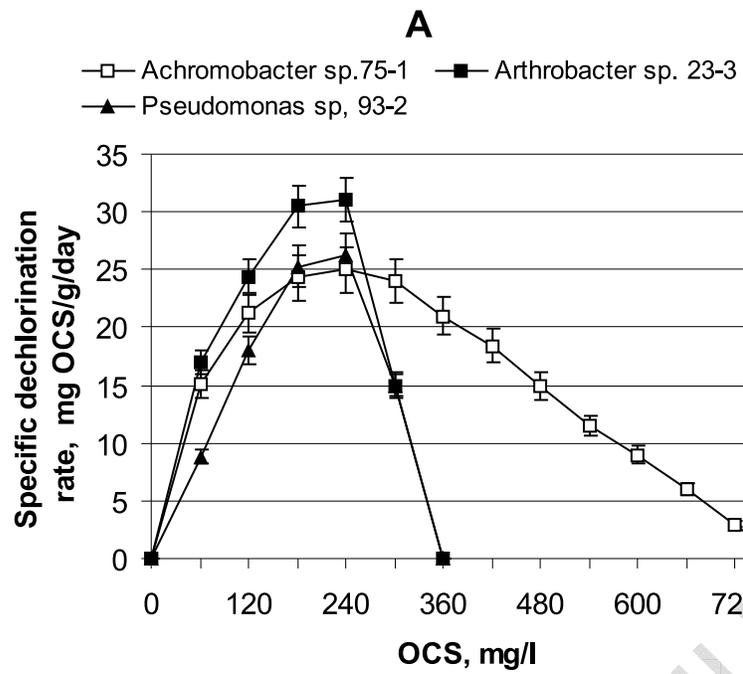


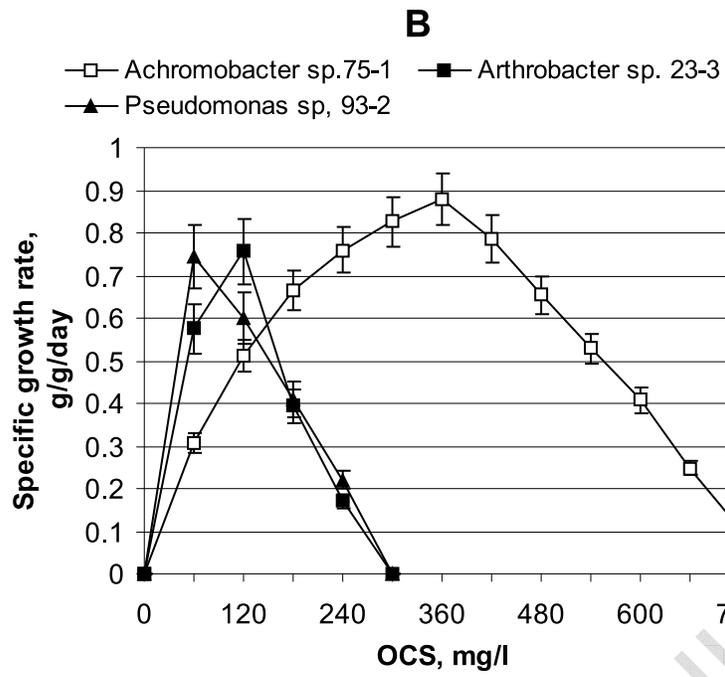


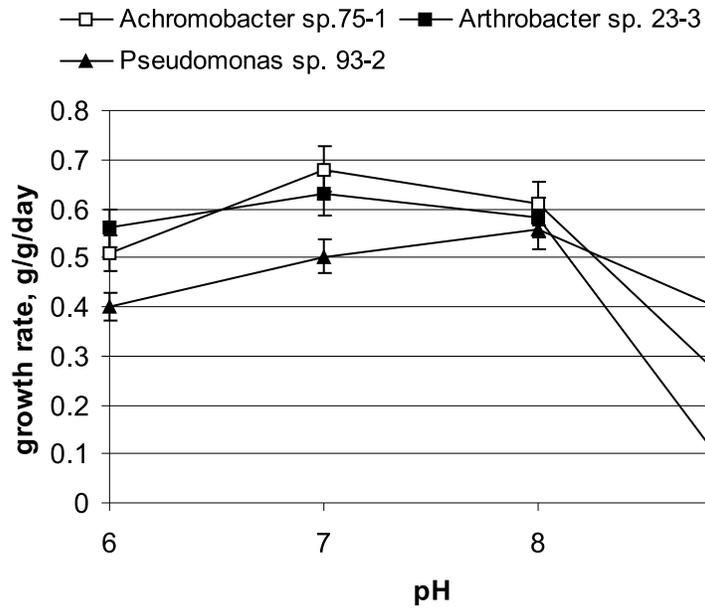


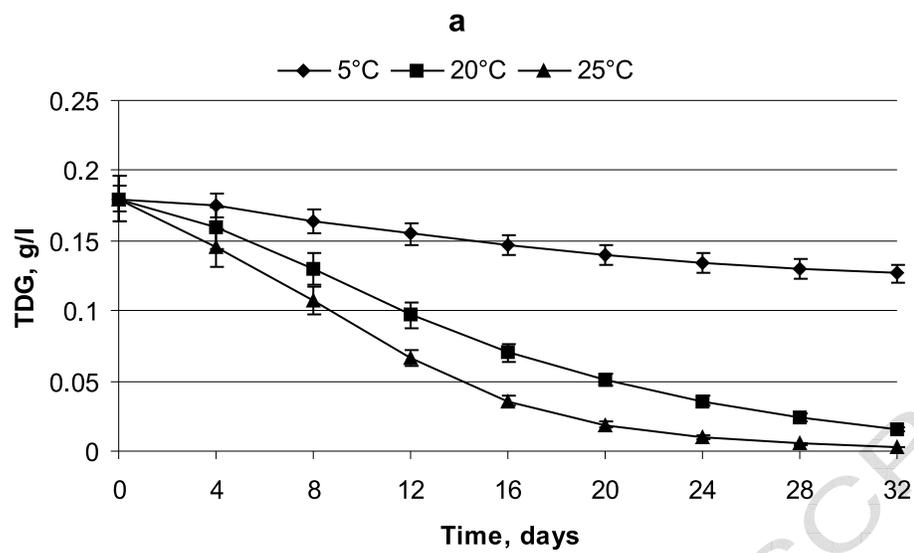


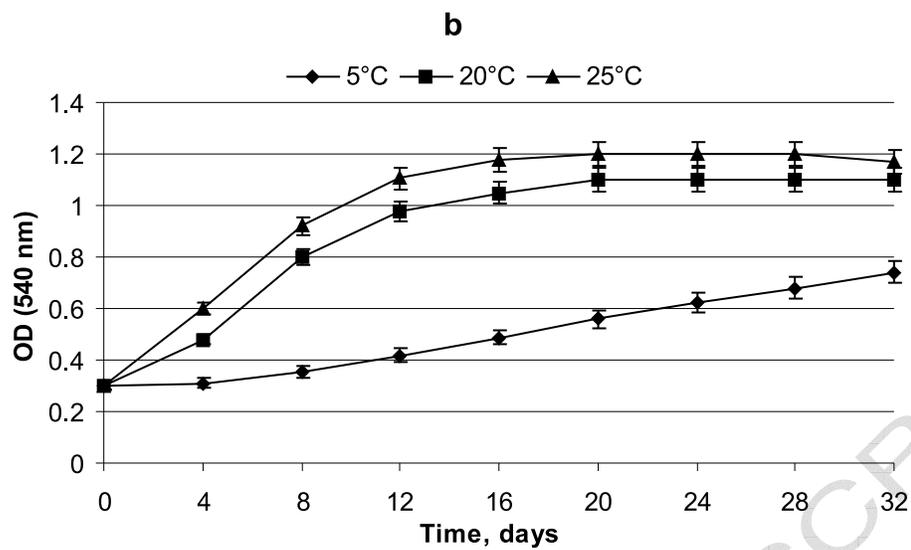


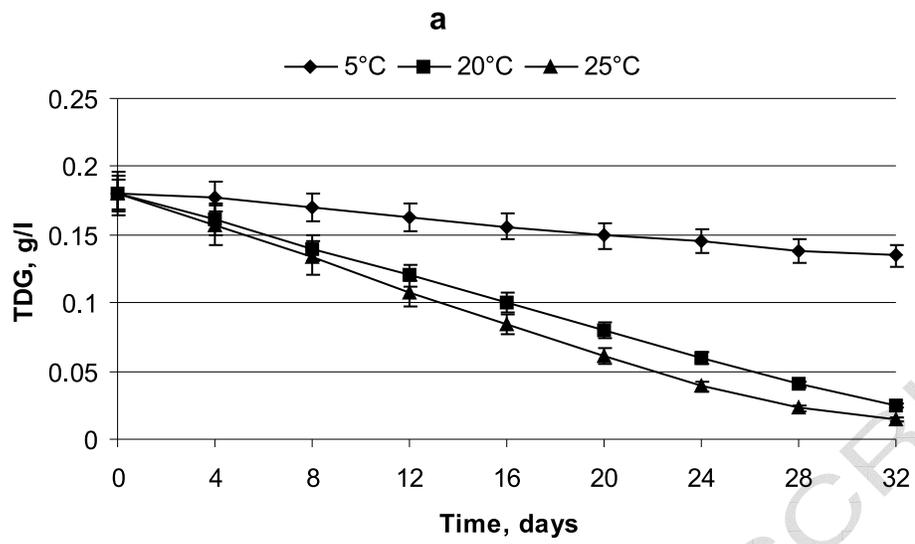


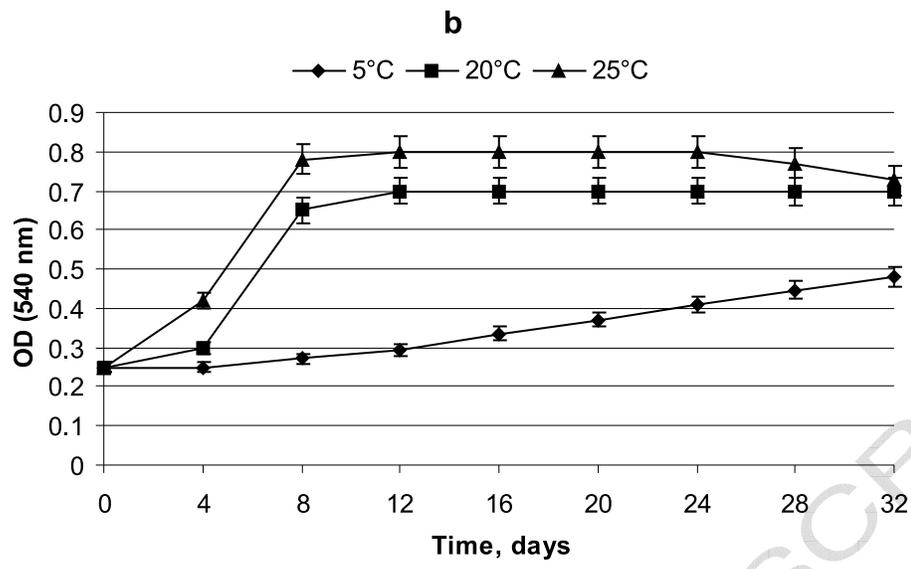


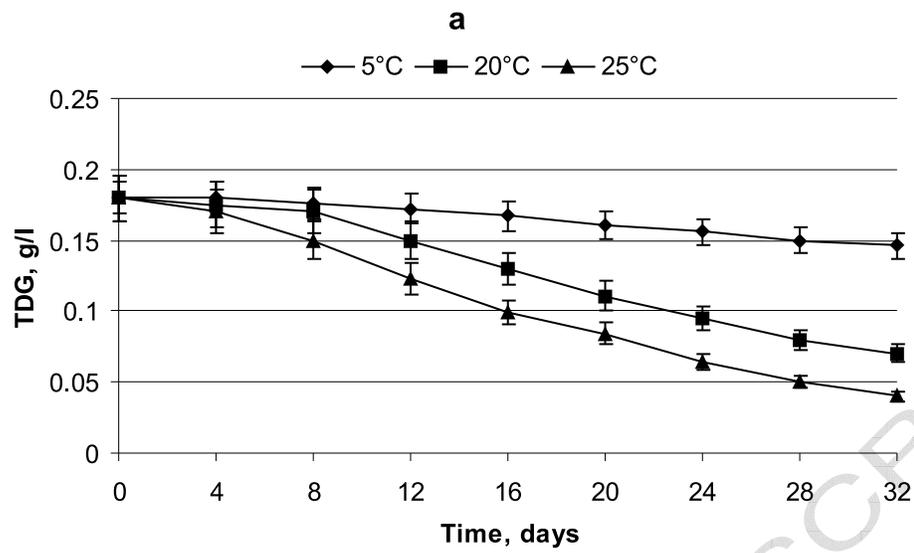


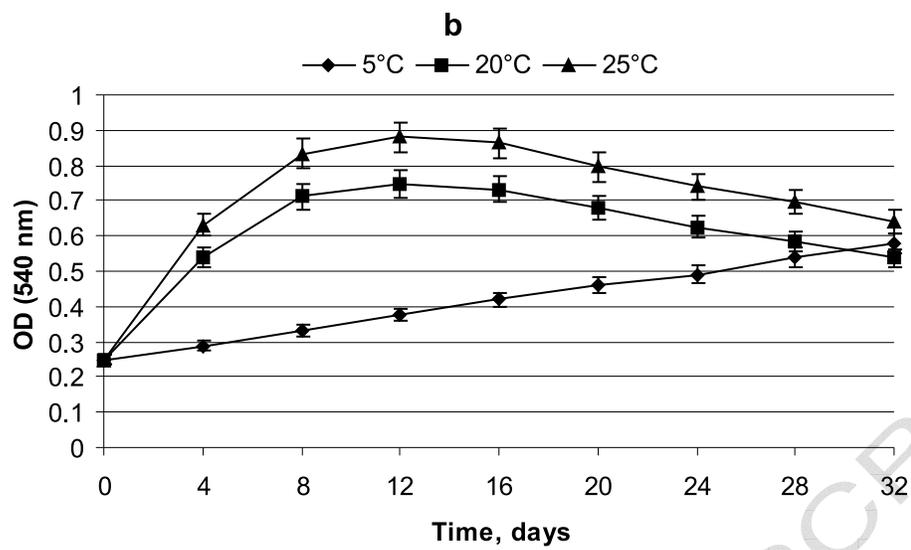


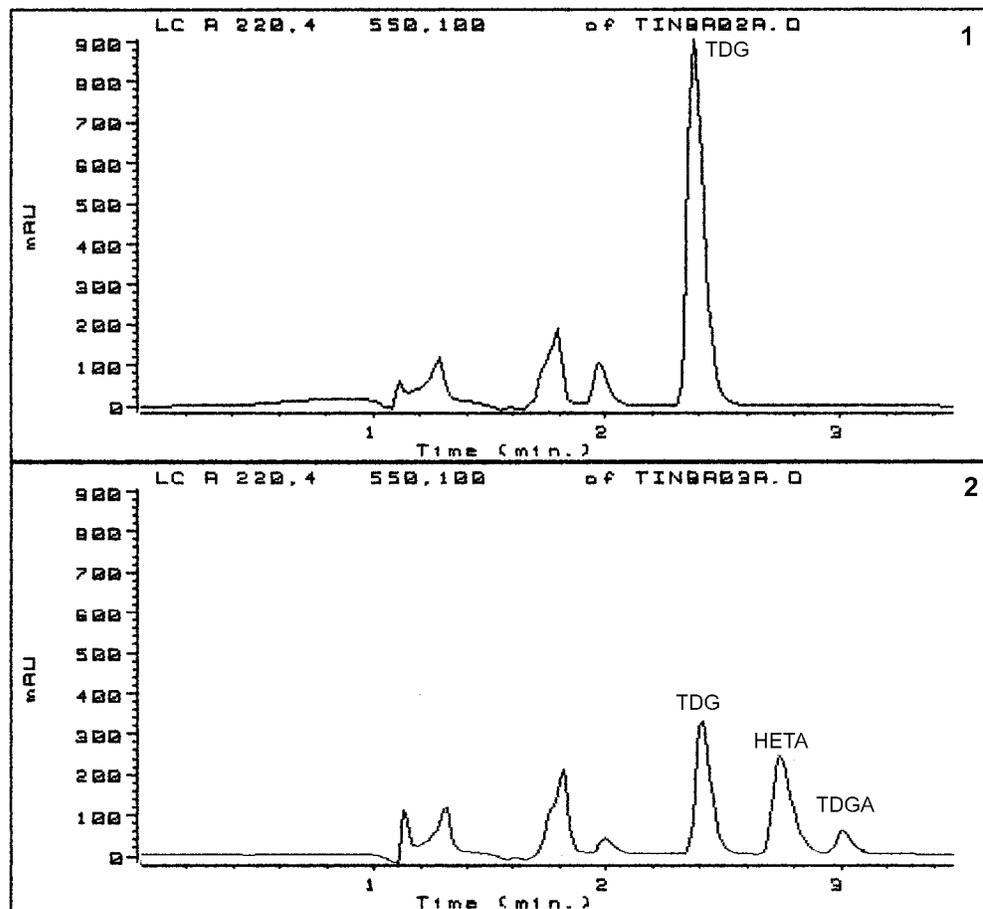


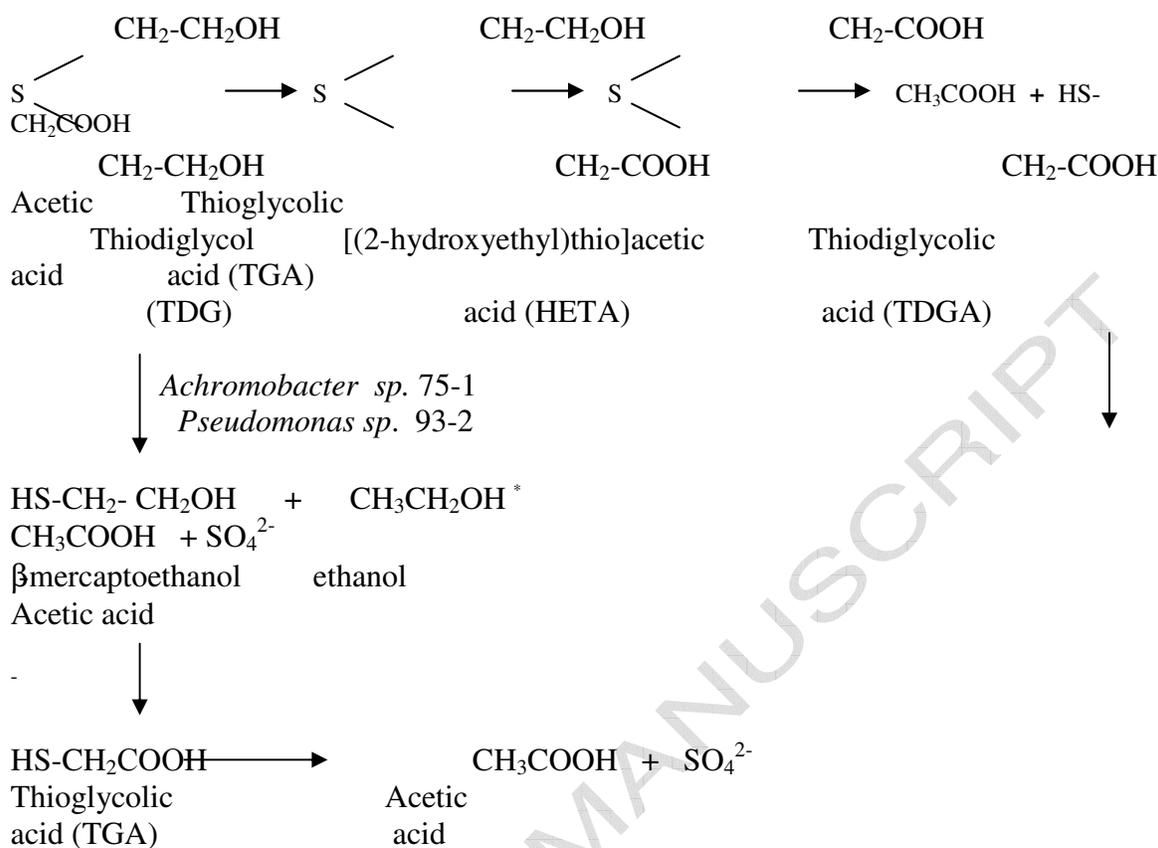












*not determined.

Table 1

Quantities of the chemical munitions and chemical war gases dumped in the southern and western Baltic Sea and Skagerrak (west of Sweden)

Area	Quantities of		Types of war gases
	chemical munitions	chemical war gases	
Bornholm Basin (E of Bornholm)	from 35,300 tons (reliable data) to 43,300 tons (unreliable data)	from 5,300 tons to 6,500 tons	Mustard gas, viscous mustard gas, Clark I, II, adamsite, chloroacetophenone (possibly, phosgene, nitrogen mustard)
Area SW of Bornholm	up to 15,000 tons (unreliable data)	2 250 tons	no data
Gotland Basin	2,000 tons	300 tons	no data
Little Belt	5,000 tons	750 tons	tabun, phosgene
Skagerrak (W of Sweden)	app. 20,000 tons (quantity not verified)	-	mustard gas (other types not verified)

Table 2

Microbiological characteristics of the Baltic Sea near-bottom water

Dumping site	Total bacterial number, $10^6/\text{ml}$	Bacterial biomass, mg/ml	Number of heterotrophs	
			Total, $10^4/\text{ml}$	MGHP-degrading microorganisms, % of total number of heterotrophs
Gotland Basin	1.3 – 4.2	1.1 – 3.0	0.6 – 2.0	up to 20
Bornholm Basin	0.7 – 5.3	0.6 – 4.9	0.5 – 3.2	up to 85
Skagerrak Strait	0.5 – 7.1	0.4 – 6.5	0.2 – 4.9	up to 98

Table 3

Taxonomic structure of the bacterioplankton in Southern Baltic Sea

Out of the dump site	Bornholm dump site
<i>Bacillus</i>	<i>Pseudomonas</i>
<i>Pseudomonas</i>	<i>Bacillus</i>
<i>Alcaligenes</i>	<i>Achromobacter</i>
<i>Sphingomonas</i>	<i>Arthrobacter</i>
<i>Achromobacter</i>	Others: <15%
<i>Flavobacterium</i>	
<i>Arthrobacter</i>	
<i>Acinetobacter</i>	
<i>Micrococcus</i>	
<i>Flectobacillus</i>	
<i>Planococcus</i>	
Others: < 25%	

Table 4

Comparative cultivation of the degrader microorganisms on the MGHPs containing medium (60 mg OCS/l and 0.18% TDG, 20°C)

Microorganisms	Static conditions		Aeration (15 mM O ₂ /l/h)	
	Biomass gain (mean ±SD), %	q _{max} * (mean ± SD), mg/g/d	Biomass gain (mean ±SD), %	q _{max} * (mean ± SD), mg/g/d
<i>Pseudomonas sp.</i> 93-2	10.4±0.6	9±1	212.1±6.4	17±2
<i>Arthrobacter sp.</i> 23-3	17.1±0.8	19±2	226.0±9.4	26±3
<i>Achromobacter sp.</i> 75-1	24.0±1.3	26±3	267.5±12.2	37±4

* q_{max} – maximum specific dechlorination rate, mg OCS/g biomass/day.

Data are means ± SD of three independent measurements.

Table 5

Effect of temperature on growth of degrader-microorganisms under static conditions

Microorganisms	Temperature, °C	Y ^a (mean ±SD), g/g	μ _{max} ^b (mean ±SD), g/g/d
<i>Achromobacter sp.</i> 75-1	5	0.37±0.02	0.07±0.01
	20	0.39±0.03	0.23±0.02
	25	0.39±0.04	0.20±0.01
<i>Arthrobacter sp.</i> 23-3	5	0.23±0.02	0.07±0.01
	20	0.29±0.02	0.34±0.03
	25	0.31±0.03	0.30±0.02
<i>Pseudomonas sp.</i> 93-2	5	0.32±0.03	0.09±0.01
	20	0.33±0.02	0.31±0.02
	25	0.35±0.03	0.35±0.03

^aY - biomass yield, g biomass produced [dry wt]/ g substrate utilized.^bμ_{max} - maximum specific growth rate, g /g biomass/day.

Data are means ± SD of three independent measurements.