LABORATORY SIMULATION OF BIODEGRADATION OF CHEMICALS IN SURFACE WATERS: CLOSED BOTTLE AND RESPIROMETRIC TEST

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ABSTRACT
Microbial degradation is the most dominant elimination mechanism of organics from the environment. For evaluation of biodegradability of pure chemicals many standardized tests are available, but no standardized procedure for assessment of biodegradability of chemicals in surface water is agreed upon. Rates of in-situ biodegradation are usually estimated in laboratory simulation where environmental factors are reproduced to some extent. The aim of our study was to compare standardised ready biodegradability assessment test (Closed bottle test) and its modifications employing the basic agreements on test conditions to simulate biodegradation in surface water. Standard test was modified using various natural river waters to simulate the natural environment in a simplified way. The impact of different types and amounts of nutrients and microorganisms on biodegradation was confirmed. The conditions in the recipient should be examined to extrapolate the results from ready biodegradability tests to real surface water.

INTRODUCTION
Numerous standardized tests are used for the control of biodegradation of pure chemicals reflecting different situations [1]. Testing methods form three levels protocol [2]:

1. Zero level tests. A variety of standardized methods corresponding to demands in ready biodegradability assessment tests exist. The common conditions are: high concentration of the test compounds as the only carbon source in the system, concentration of the inoculum less than 30 mg SS/l (10⁴ - 10⁸ cells/l), added mineral medium as source of nutrients and buffer system, neutral pH (7.2/7.4) and constant temperature (20°C or 25°C (MITI test)) [1]. The aim of performing these tests is to provide information on biodegradability of a substance under most common environmental conditions. Biodegradation is monitored with use of a summary parameter (O₂ consumption, CO₂ production, etc.) for 28 days and presented as biodegradation curves. The substance is ready biodegradable if at least 60 % of initially added test compound measured as O₂/CO₂ is removed. Time after initiation of mineralization (tL, lag time) and maximum level of degradation should be no longer than 10 days (10 days window). Degradation rate (k),
derived from the 10 days window with presumption of first order kinetics, should be higher than 0.14 day⁻¹. Ready biodegradable substances are assumed to degrade in the environment.

2. **First level tests.** Potential biodegradability of chemical substances are tested in inherent biodegradability assessment tests. If the substance is not degradable at this level, it can be proclaimed with great certainty as persistent.

3. **Second level tests.** Tests for ready and inherent biodegradability were designed to determine if the chemical has the potential to mineralize. These tests were not intened to predict the behaviour of chemicals in the various compartments of the aquatic environment [1]. This is the purpose of the simulation tests. Standardised simulation tests have been established only for the activated sludge system [3]. Many papers describe simulations of a wide variety of environments (surface waters, sediments, soil, etc.), but no standardised methods have been agreed upon yet.

It is difficult to estimate biodegradation rates in surface water because of the influence from many transformations and transport processes (dilution, sorption, etc.) which besides biodegradation fundamentally contribute to the disappearance of the chemical [4]. At the same time, it would entail a lot of time and money to test biodegradation of all chemicals in the field. Due to these difficulties, rates of *in-situ* biodegradation are usually estimated from the results of laboratory simulation. However, a lot of environmental factors (seasonal/daily variations in physico-chemical conditions, nutrient concentration, presence of other organics, etc.) are not sufficiently reproduced under laboratory conditions. Nevertheless, the question arises as to whether results of tests for ready biodegradability can be used for predictive purposes. Nutrient sufficiency and high substrate and microbial concentrations are main test system limitations when the prediction of environmental degradation from ready tests is considered [4]. The use of natural river waters without addition of nutrients and inoculum in ready tests combines conditions from tests and surface waters.

**MATERIALS AND METHODS**

**Test Compounds.** All biodegradability assessment tests were performed with two chemicals, both were of analytical grade, obtained from Kemika (Croatia): formaldehyde (CHOH) and propylene glycol (C₃H₆O₂). They are widely used in synthesis of different resins and often enter surface waters. Stock solutions of chemicals with concentration of 100 mg/l were prepared just prior to the test. Appropriate volume was introduced to achieve test concentrations: 4.0 mg/l formaldehyde (Theoretical Oxygen Demand (ThOD) = 1065 mg/g; Theoretical Carbon Dioxide Production (ThCO₂) = 1467 mg/g) and 3.0 mg/l propylene glycol (ThOD = 1425 mg/g; ThCO₂ = 1808 mg/g) in all biodegradability tests.

**Toxicity measurements.** Possible false persistency evaluation was prevented with toxicity testing. Toxicity of both chemicals was measured and calculated according to the test for inhibition of oxygen consumption by activated sludge [5]. Tests were performed with low concentration of microorganisms (100 mg SS/l) to approach conditions in surface waters.
Test medium. In standardized closed bottle tests [6], deionized water with adequate salts (introducing nutrients and buffer system) was used (synthetic medium). In modified biodegradation tests seven river waters were applied: two from different rivers (R1, R2) and five sampled from third river R3 at five sampling sites: R3S1, R3S2, R3S3, R3S4 and R3S5. River waters were random grab sampled, for biodegradation studies samples were stored at +4°C for not more than 5 days. BOD₅ [7] was determined immediately after sampling. For COD [8], orthophosphate, N-Kjeldahl, ammonia, nitrite and nitrate determination [9], samples were frozen at -28°C.

Due to the high variety of microfauna and nutrition components in different river waters or even in the same river and the same place at different time, river waters were ranked into four groups. Determination of same common characteristics of river waters helps us to get overall understanding of the impact of river water quality on biodegradation. The classification of river waters is based on nutrient load [10]: oligotrophic surface water (<0.01 mg/l P), mesotrophic surface water (0.01 - 0.03 mg/l P), eutrophic surface water (>0.03 mg/l P) and hypereutrophic surface water (>0.1 mg/l P).

Biodegradation measurements. Tests were run to estimate the impact of different types of river waters on the rate and final level of mineralization. Ready biodegradability assessment tests were performed in two ways, using different measurement techniques:

1. In BOD bottles standard closed bottle test [6] and tests with two river waters (R1,R2) were done. In standard tests all solutions (1 ml/l) and inoculum were added to the test substance. Secondary effluent from a laboratory municipal wastewater treatment plant was used as inoculum. Influent of the treatment unit was constituted of 130 mg/l peptone, nutrients (0.9 mg/l P as KH₂PO₄), 70 vol. % of distilled water and 30 vol. % of municipal sewage. The appropriate amount of inoculum was estimated using a blank test: with addition of 0.5 ml of the effluent to 1 l of test medium, the oxygen depletion was less than 1.5 mg/l (as required by standard procedure). The bottles were completely filled with prepared mixture, sealed and incubated in darkness up to 28 days (20±2°C). All tests were run in duplicates, besides bottles with test compounds and blanks, activity of inoculum was checked simultaneously with the reference compound (5 mg/l sodium acetate).

The same test design was applied in the tests with river waters R1 and R2. Biodegradation was estimated with the same concentrations of the test compounds as in the standardised test: with addition of nutrients (solution A [6], containing N and P), with addition of inoculum (0.5 ml/l secondary effluent), and with addition of both, nutrients and inoculum, to check their impact on biodegradation of tested substances. In all cases biodegradation was followed by determining oxygen concentrations (measured by WTW OXI 91 oxygen electrode) as mg/l every 5 days.

2. In closed respirometer (Micro OXymax Respirometer with closed circuit mode, Columbus Instruments, Ohio) biodegradation studies with river waters sampling at 5 sites downstream river water R3 were performed. Test substances were added in the river water in the same concentrations as in closed bottle tests.
No nutrients and inoculum were added. 250 ml of mixture was put in the measuring chamber of the respirometer. Constant temperature (20±1°C) was maintained. Cumulative consumption of O₂ and CO₂ production (mg/l) were measured simultaneously once a day up to 28 days.

**Biodegradation calculation.** Measured depletion of oxygen concentration in the test with test component was corrected for the blank value and divided with ThOD to calculate % of degradation. The same procedure was applied calculating biodegradation from CO₂ production (respirometric measurements). Biodegradation curves were plotted as % of biodegradation versus time for each vessel. Mean value of replicates was calculated [6]. average biodegradation curves were plotted. Factors, characterising biodegradation (lag phase tₜₜ (days) - time to reach 10% of theoretical expected degradation; degradation time tₜ (days) - time after tₜ needed to reach 90% of final mineralization plateau; final level (%) of substances removal, were read from the average degradation curves. Rates of biodegradation (k, day⁻¹) were calculated using equation /1/ with assumption of first order degradation kinetics [11].

\[
\ln \left( \frac{c_o}{c} \right) = k \cdot t \text{ (day)}^{-1}
\]

**RESULTS AND DISCUSSION**

**Analysis of river waters.** Physico-chemical analysis of surface waters, used in biodegradation tests is presented in Table 1.

<table>
<thead>
<tr>
<th>River Water</th>
<th>R1</th>
<th>R2</th>
<th>R3S1</th>
<th>R3S2</th>
<th>R3S3</th>
<th>R3S4</th>
<th>R3S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance from the spring (km)</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>2.1</td>
<td>5.2</td>
<td>6.7</td>
<td>10.7</td>
</tr>
<tr>
<td>COD (mg/l)</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>1.2</td>
<td>1.6</td>
<td>5.3</td>
<td>7.3</td>
<td>8.5</td>
</tr>
<tr>
<td>BOD₅ (mg/l)</td>
<td>&lt; 0.5</td>
<td>&lt; 0.5</td>
<td>&lt; 0.5</td>
<td>0.7</td>
<td>0.7</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>N-Kjeld. (mg/l)</td>
<td>/</td>
<td>/</td>
<td>0.21</td>
<td>0.21</td>
<td>1.87</td>
<td>1.14</td>
<td>0.66</td>
</tr>
<tr>
<td>NH₃-N (mg/l)</td>
<td>0.22</td>
<td>/</td>
<td>&lt; 0.02</td>
<td>&lt; 0.02</td>
<td>&lt; 0.02</td>
<td>0.21</td>
<td>0.21</td>
</tr>
<tr>
<td>NO₂-N (mg/l)</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>0.015</td>
<td>0.012</td>
</tr>
<tr>
<td>NO₃-N (mg/l)</td>
<td>0.07</td>
<td>1.10</td>
<td>0.83</td>
<td>0.81</td>
<td>1.15</td>
<td>1.15</td>
<td>1.06</td>
</tr>
<tr>
<td>PO₄³⁻-P (mg/l)</td>
<td>&lt; 0.005</td>
<td>0.100</td>
<td>0.016</td>
<td>0.186</td>
<td>0.046</td>
<td>0.033</td>
<td>0.020</td>
</tr>
</tbody>
</table>

R Classification | OT | HET | MT | HET | ET | ET | MT
---|----|-----|----|-----|----|----|----
R ....River Water; S ....Sampling Site; / ....Analysis was not performed
OT ....Oligotrophic; MT ....Mesotrophic; ET ....Eutrophic; HET ....Hypereutrophic

According to P content, only river water R1 was oligotrophic. River water R2 was hypereutrophic. The same was R3S2, while other waters sampled downstream the third river were mesotrophic (R3S1, R3S5), and eutrophic (R3S3, R3S4). Organic pollution (COD, BOD₅, N-org.) was rising from R3S1 to R3S3 as a consequence of municipal wastewaters, released from a small town (3000 inhabitants) located near river R3 (between R3S2 and R3S3). Changes in nitrogen components indicated river selfpurification (from R3S3 to R3S5). Synthetic medium, used in standardized test was hypereutrophic (>0.1 mg/l P).
Toxicity of test compounds. EC_{10.3hrs} (10% inhibition of oxygen consumption after 3 hours of exposure) of formaldehyde is 9.2 mg/l (Standard Deviation = 2.3, N = 3) [9]. Its concentration used in biodegradation tests was lower (4 mg/l). At the same time no inhibition of O\textsubscript{2} consumption/CO\textsubscript{2} production was noticed during biodegradability testing and we assumed that toxic effect is avoided. The same was found with propylene glycol (EC\textsubscript{10.3hrs} = 455 mg/l, concentration used in biodegradation studies was 3.0 mg/l).

Biodegradation of formaldehyde. Biodegradation curves of formaldehyde in standard closed bottle test (SCBT) and its modifications (river waters R1 and R2), obtained from O\textsubscript{2} consumption in BOD bottles, are presented in Figure 1. Additional closed bottle tests were performed with oligotrophic water R1, where nutrients N and P (solution A) and inoculum were added to check the impact of nutrients and sludge microorganisms on formaldehyde degradation. Results (t\textsubscript{L}, t\textsubscript{W}, final levels of mineralization) are in Figure 2.

As seen in Figure 2, addition of nutrients to oligotrophic river water R1 reduces the lag phase, and increases degradation rates (from k=0.09 to 0.55 day\textsuperscript{-1}), while the final level of degradation remains constant (18%). Addition of inoculum reduces the lag phase and does not affect the mineralization rate. Addition of both, nutrients and inoculum, increases final level of degradation (from 18 to 46%), while degradation rate remains nearly constant (0.07 day\textsuperscript{-1}). The same pattern of degradation was observed with measurements in respirometer with samples from river R3. In Table 2 are biodegradation results (average, minimal and maximal values) for formaldehyde represented as lag times, degradation times, rates of degradation and final levels of degradation in waters from 5 sampling sites downstream river R3 (calculated from O\textsubscript{2} consumption and CO\textsubscript{2} production).
Table 2. \( t_1, t_w, k \) and final level of degradation (deg) obtained from \( O_2 \) and \( CO_2 \) measurements in respirometers, with 4 mg/l of formaldehyde in river waters R3S1, R3S2, R3S3, R4S4 and R5S5.

<table>
<thead>
<tr>
<th>RW Class</th>
<th>( t_1 ) (day)</th>
<th>( t_w ) (day)</th>
<th>( k ) (day(^{-1}))</th>
<th>Deg (%)</th>
<th>( t_1 ) (day)</th>
<th>( t_w ) (day)</th>
<th>( k ) (day(^{-1}))</th>
<th>Deg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3S1 MT</td>
<td>1.2 ± 0.5</td>
<td>2.0 ± 0.4</td>
<td>0.20 ± 0.01</td>
<td>83 ± 3</td>
<td>2.0 ± 0.5</td>
<td>6.6 ± 0.5</td>
<td>0.26 ± 0.02</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>R3S2 HET</td>
<td>6.5 ± 0.9</td>
<td>1.8 ± 0.2</td>
<td>0.25 ± 0.09</td>
<td>50 ± 3</td>
<td>1.8 ± 0.2</td>
<td>9.0 ± 0.7</td>
<td>0.04 ± 0.04</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>R3S3 ET</td>
<td>6.0 ± 1.0</td>
<td>3.0 ± 0.2</td>
<td>0.44 ± 0.03</td>
<td>80 ± 1</td>
<td>1.6 ± 0.2</td>
<td>8.0 ± 1.0</td>
<td>0.10 ± 0.01</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>R3S4 MT</td>
<td>0.5 ± 0.2</td>
<td>7.5 ± 0.6</td>
<td>0.20 ± 0.01</td>
<td>83 ± 3</td>
<td>1.0 ± 0.1</td>
<td>7.1 ± 0.9</td>
<td>0.19 ± 0.03</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>R3S5 MT</td>
<td>0.5 ± 0.3</td>
<td>7.5 ± 0.1</td>
<td>0.18 ± 0.01</td>
<td>82 ± 3</td>
<td>0.7 ± 0.2</td>
<td>7.0 ± 0.9</td>
<td>0.15 ± 0.01</td>
<td>83 ± 5</td>
</tr>
</tbody>
</table>

RW - River Water: O1...Oligotrophic; MT...Mesotrophic; ET...Eutrophic; HET...Hypereutrophic

Biodegradation of propylene glycol. Biodegradation curves of propylene glycol (3 mg/l) in standard closed bottle test (SCBT) and in R1 and R2, measured as oxygen consumption in BOD bottles are shown in Figure 3. The impact of nutrients and inoculum to propylene glycol biodegradation in oligotrophic R1 was checked and results in form of \( t_1, t_w \) and final level of degradation are presented in Figure 4.

Considering results of standardised test, propylene glycol was not a readily biodegradable substance (\( k=0.14 \) day\(^{-1}\); \( t_w=10 \) days). The most intensive degradation occurs in hypereutrophic water R2 (\( k=0.18 \) day\(^{-1}\)), while in oligotrophic R1 only 37 % of the substance degraded after substantial lag period. As could be seen in Figure 4, addition of nutrients to oligotrophic R1, decreases \( t_1 \) and increases final level of degradation and degradation rate (from \( k=0.22 \) to 0.27 day\(^{-1}\)). Inoculated microorganisms reduced \( t_1 \), increased % of degradation, while degradation rates were lower (0.07 day\(^{-1}\)). This phenomenon could be explained as impact of organic matter released from biomass present [13].

Biodegradation (average, maximal and minimal values) of propylene glycol in 5 river waters downstream R3 in forms of \( t_1, t_w \), degradation rates (k) and final level of degradation (deg) are presented in Table 3, as calculated from \( O_2 \) and \( CO_2 \) measurements.
Figure 3. Biodegradation of propylene glycol, from O₂ readings in BOD bottles (Standard closed bottle test (SCBT); tests with river waters R1, R2).

Figure 4. \( t_L, t_w, \% \) of degradation obtained from studies with 3 mg/l propylene glycol:


Table 3. \( t_L, t_w, k \) and final level of degradation (deg) obtained from O₂ and CO₂ measurements in respirometer, with 3 mg/l of propylene glycol in river waters R3S1, R3S2, R3S3, R3S4 and R3S5.

<table>
<thead>
<tr>
<th>RW Class</th>
<th>O₂ Consumption</th>
<th>CO₂ Production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_L(\text{day}) )</td>
<td>( t_W(\text{day}) )</td>
</tr>
<tr>
<td>R3S1 MT</td>
<td>4.0±0.8</td>
<td>7.0±0.2</td>
</tr>
<tr>
<td>R3S2 HET</td>
<td>2.0±1.0</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>R3S3 ET</td>
<td>1.2±0.5</td>
<td>12.0±1.0</td>
</tr>
<tr>
<td>R3S4 ET</td>
<td>1.0±0.4</td>
<td>3.0±0.8</td>
</tr>
<tr>
<td>R3S5 MT</td>
<td>1.1±0.7</td>
<td>4.0±0.6</td>
</tr>
</tbody>
</table>


Propylene glycol was degraded quickly in all 5 river waters. Final levels of degradation were comparable. \( t_L \) and \( t_w \), calculated from CO₂ production were longer than calculated from O₂ measurements, probably as a consequence of slower CO₂ release (pH at the end was 7.4). Minimal difference between biodegradation in meso- and eutrophic waters R3S3 to R3S5 can be interpreted as an impact of organic pollution (COD: 5.3-8.5 mg/l) and related microorganisms present (probably adapted). Generally, presented river water classification, based on nutrient contents, is accurate enough to distinguish between biodegradation levels/rates in oligo-, hyper- and eutrophic surface waters, while the differences between meso-and eutrophic waters at higher organic loads are not clear enough.

CONCLUSION
Accomplished experiments confirmed low degradation potential of standardized closed bottle test, which belongs to the highest end of the biodegradability testing stringency scale. This test alone is not sufficient to assure appropriate degradation characterization of the substance and it must be supplemented with one of
the other ready tests. It was clearly observed, that degradation rates and lag phases are dependent on type of river water used (oligotrophic/eutrophic) and thus quantity and quality of present nutrients, bacteria and organics. Generally, nutrients increase degradation rates and shorten lag time. It was confirmed, that specific characteristics of surface waters should be taken into account, when extrapolating results of standardized ready biodegradability assessment tests to field conditions.

REFERENCES


