EFFECTS OF HYDROGEN SULFIDE TO VIBRIO FISCHERI, SCENEDESMUS VACUOLATUS, AND DAPHNIA MAGNA

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Abstract—The effects of hydrogen sulfide (H$_2$S) were tested in three ecotoxicological tests in order to evaluate its confounding potential in assessment of pore water and groundwater toxicity. The luminescent bacteria Vibrio fischeri, the water flea Daphnia magna, and the microalgae Scenedesmus vacuolatus often are part of a biotest battery. A new technique for the synthesis of hydrogen sulfide solutions of defined concentrations using an electrochemical generator instead of sodium sulfide solutions was used. Because hydrogen sulfide is volatile, the loss rate of H$_2$S was studied over time to enable estimation of the mean test concentrations over the whole test duration. Loss rates were calculated to be 13 ± 6% after 30 min, and 39 ± 11% and 43 ± 16% after a 24- and 48-h exposure time, respectively. Sensitivities of the test organisms in terms of median effective concentration (EC50), corrected for the above loss rates, varied from 0.28 to 0.0036 and 0.055 mM for the luminescent bacteria, the crustacea, and the algae, respectively. A species-sensitivity distribution using EC and mean lethal concentration literature data for marine and freshwater crustaceans and phytoplankton showed a medium sensitivity of the water flea D. magna, though the bacteria V. fischeri and the algae S. vacuolatus were among the least-sensitive group of organisms. This demonstrates that only the algae and the bacteria are easy to use in the assessment of toxicity of matrices with H$_2$S concentrations above 0.06 mM.

Keywords—Hydrogen sulfide toxicity Vibrio fischeri Daphnia magna Scenedesmus vacuolatus Species-sensitivity distribution

INTRODUCTION

Hydrogen sulfide is known as a disturbing or confounding factor in sediment toxicity biotests [1] and other ecotoxicological assays [2,3]. A good deal of evidence has been produced about the influence of hydrogen sulfide to sewage treatment plant wastewater toxicity [4,5]. Because hydrogen sulfide is toxic (see [2,6,7]) and readily forms metal-sulfides, it can cover up possible toxic effects of other substances and metals [8,9]. Typically, hydrogen sulfide or elemental sulfur are excluded rather than included in risk-assessment or toxicity evaluation studies, because H$_2$S is volatile and the concentration of H$_2$S in solution is highly dependent on parameters like pH and oxygen and salt content. Thus, different techniques are recommended by the toxicity identification and evaluation (TIE) protocols to eliminate pH- and oxygen-dependent substances such as the acid and heat volatile sulfide species of H$_2$S by using the techniques of pH adjustment, heating of sediment, or aeration of samples [1,10].

During a surveillance and monitoring study investigating possible toxic components of complex contaminated groundwater [11], hydrogen sulfide was identified as a possible confounding factor because it reached concentrations of up to 0.088 mM. With the available literature data, it was not possible to exclude that effects of groundwater seen in different ecotoxicological biotests (marine bacterium Vibrio fischeri, the crustacean Daphnia magna, and the micro algae Scenedesmus vacuolatus) was not based solely on hydrogen sulfide.

Field studies and laboratory tests already have demonstrat-ed the toxicity of hydrogen sulfide to different groups of organisms such as crustaceans, algae, fish, and other vertebrates. Most authors identify the primary cause of toxic effects as the immediate binding of hydrogen sulfide to the enzyme cytochrome-c-oxidase (EC 1.9.3.1) or other metallo- and disulfide-containing proteins [6,12]. Others have hypothesized the un-coupling of mitochondrial respiration via the opening of the mitochondrial permeability transition pore as an additional mechanism of toxicity resulting in depletion of intracellular levels of adenosine triphosphate [13].

Three charge states exist that occur naturally in the environment: H$_2$S, HS$^-$, and S$^2$$. Under physiological pH, only the first two charged species (hydrogen sulfide and the sulfide anion) are found. Many authors have published research showing that the toxicity mostly is due to hydrogen sulfide (H$_2$S) because hydrogen sulfide is known as a very poisonous gas that, for example, leads to pulmonary edema when administered orally [7], and that often is found in sewage treatment plants and under other anoxic conditions. Other authors infused or used different solutions of NaSH [14], or more often Na$_2$S, and implied that HS$^-$ was the toxic component. Some authors do not differentiate at all between the three species [14]. The reason for this is the inability to prove that the toxicity is based on one or the other of the two charged species. The intracellular effects of H$_2$S/HS$^-$ usually are thought mainly to be based on the inhibition of the mitochondrial cytochrome-c-oxidases, although several other modes of actions are discussed also in Reifenstein et al. [15]. Because of the pKs values of about 7.01 [16], the intracellular concentrations of H$_2$S and HS$^-$ will be equally high, so that it is difficult to argue that one or the
other (hydrogen sulfide or the sulfide anion) solely is responsible for the toxic effects in cells or organisms. Thus, one could speak equally about HS\(^-\) toxicity, H\(_2\)S toxicity, or a joint toxicity of both species. However, because HS\(^-\) is charged, it is unlikely that it will diffuse easily into the cells. In contrast, H\(_2\)S is much more capable of permeating the cell membranes [17], so cytochrome-c-oxidase inhibition or other effects that are seen in the cells essentially will depend on the extracellular concentration of H\(_2\)S and not HS\(^-\). Because it is still difficult to quantify the actual intracellular HS\(^-\) concentrations, we thought it more appropriate to assign the effects to the extracellular H\(_2\)S concentration. This is a problem that often is seen in (eco-)toxicological research where the intracellular concentration (i.e., the internal and effective dose of a substance) is unknown. Therefore, for example, it has become common practice to write about ammonia toxicity instead of NH\(_3\) or NH\(_4\)\(^+\) toxicity, or chlorophenol toxicity instead of the toxicity of the charged species of chlorophenol.

In addition, the mode and site of action of H\(_2\)S/HS\(^-\) in the cells is not yet completely understood. Because of the above assumptions, we did not try to differentiate between the effects of H\(_2\)S/HS\(^-\), but assigned our data to the extracellular concentrations of H\(_2\)S that we quantified.

Because hydrogen sulfide often is present in both polluted and unpolluted sediments/pore water and other environmental samples, its toxic effect may cover up effects generated by anthropogenic contaminations in the environment. Thus, the toxic effect of hydrogen sulfide may make the source of toxicity in an analyzed environmental sample more difficult to identify when the sample’s toxicity is due to contaminations, metals, or other confounding factors such as ammonia [2,18].

No information was available about the toxic effects of hydrogen sulfide to the three test organisms together as a tool for toxicity evaluation of sediments and groundwater. Luminous bacteria, the crustacea *Daphnia* sp., and microalgae such as *S. vacuolatus* often are part of a biotest battery. The specific objectives of this study were to determine dose-response relationships of hydrogen sulfide to organisms of different genera used in standard ecotoxicological aquatic test batteries. In addition, the behavior of hydrogen sulfide due to its volatility was studied to enable the correction of the assumed losses through volatilization and oxidation. Consequently, the calculation of influences on the toxic effects of hydrogen sulfide in environmental samples is possible. Additionally, a differentiation between toxic effects generated by contaminant water and by hydrogen sulfide can be accomplished. Because the sensitivity of the three test organisms relative to other organisms was not known, a species-sensitivity distribution (SSD) was calculated by using EC50 datasets of marine and freshwater crustaceans and different phytoplanktonic species. Those datasets serve as a mean to see whether the luminous bacteria *V. fischeri*, the water flea *D. magna*, and the microalgae *S. vacuolatus* are suitable organisms for a cumulative risk assessment.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals and media were used and prepared of at least pro analysi purity substances. Substances for the buffers and the culture media (*Daphnia* culture medium, [19] and algal growth medium [20]) were from Merck (Darmstadt, Germany).

The reagents for the cultivation of luminous bacteria also were of analytical-grade quality and purchased from Merck if not annotated otherwise. The growing medium for platings contained: NaCl (513 mM), Na\(_2\)HPO\(_4\) (44.2 mM), K\(_2\)HPO\(_4\) (12 mM), MgSO\(_4\) (0.24 mM), (NH\(_4\))\(_2\)HPO\(_4\) (3.78 mM; Aldrich, Germany), glycerol (100%) 3 mL L\(^{-1}\), peptone from casein 5 g L\(^{-1}\), yeast extract (Sigma, Seelze, Germany) 0.5 g L\(^{-1}\), and 12 g L\(^{-1}\) agar (excluded in the liquid culture). Glycerol was increased to 25% (v/v) in the liquid culture for storage at –80°C. The solution was titrated with NaOH (1 M) to pH 7.0 (pH 7.2 for liquid culture) and autoclaved.

**Hydrogen sulfide synthesis**

For the synthesis of hydrogen sulfide solutions of defined concentrations, an electrochemical generator was used (Model G200e, Analysensystemtechnik, Rostock, Germany). This generator commonly is used for the online synthesis of standard solutions of H\(_2\)S/sulfide for the calibration of H\(_2\)S amperometric sensors. The synthesis of hydrogen sulfide solutions was executed in the following way: 0.01 N H\(_2\)SO\(_4\) (carrier solution) in Millipore\(^®\) water (MilliQ UFPlus, Millipore, Eschborn, Germany) was degassed in an ultrasonic water bath for 15 min, followed by 30 min of additional sparging with nitrogen to eliminate any remaining oxygen. Because of the fast reoxygination of the degassed water, the predegassed carrier solution was pumped through another external online degassing module (Analysensystemtechnik) to the H\(_2\)S generator. The desired concentration of the hydrogen sulfide solution was specified using a standard flow rate of 1.7 ml per min and a customized working current of 0.01 to 3 mA generating hydrogen sulfide solutions with concentrations from 0.6 \(\mu\)M to 0.6 mM. The resulting H\(_2\)S solution was mixed with a doubled, concentrated and buffered test medium in a 1:2 ratio. The mixing was accomplished via a flexible-tube pump (Model Ismatec Reglo, Laboratoriumstechnik, Wertheim-Mondfeld, Germany) and a T-branch connecting the outgoing lines of the H\(_2\)S generator with the tubes of the flexible-tube pump (Fig. 1). To control the final resulting hydrogen sulfide concentration in the H\(_2\)S/buffered test solution, an amperometric H\(_2\)S-multisensor (Analysensystemtechnik) of the appropriate range (0.29–88, 1.5–290, or 15–1470 \(\mu\)M) was connected to the outflow of the generator/buffer line. The synthesized, buffered H\(_2\)S solution then was used immediately in the respective biotests. The concentration of the remaining hydrogen sulfide was controlled after the respective incubation (i.e., test time in the biotests) using an H\(_2\)S sensor for the required range of H\(_2\)S concentrations.
Buffer selection

Different buffers were tested to stabilize the pH of the test media after mixing with hydrogen sulfide solutions. The stabilized pH is supposed to be between 6.2 and 6.8. Possible pH-dependent effects on test organisms were checked in respective controls. Tested buffers all were prepared and used at 20°C (except the Lumistrox© test, which was run at 15°C; B. Lange, Düsseldorf, Germany) and had a concentration of 40 mM with an ionic strength of 690 mM (4% NaCl) in the Lumistrox test and 154 mM in the algae and Daphnia test. Buffers tested for their usefulness in the biotests were: N-(2-Acetamido)-2-amoethansulphonic acid (ACES) buffer (pH 6.9), phosphate buffer (pH 6.9), N-(tris[hydroxymethyl]-methyl)-glycine (Tricine) buffer (pH 7.2), and 3-(N-morpholino)propane sulphonylic acid (MOPS) buffers (pH 6.7 and 7.2). Of the four buffer substances, the ACES buffer stabilized the pH best. Within the Lumistrox test, the pH to be reached after addition of 2× concentrated ACES-buffered medium or with ACES-buffered double-distilled water. The pH in the acute test system then was calculated using Equation 1. The pH-dependent effects on test organisms were checked in respective controls. Tested buffers all were prepared and used within the Lumistrox test system. In contrast to the Lumistrox test, closed vials and the algae test always were above 6.4 and 6.5, respectively. Controls for the respective tests consisted of two types of controls using the ACES-buffered test media: With and without pH-adjustment to the resulting pH in the actual test vials after addition of H₂S.

Loss rates of H₂S in the used test systems

The concentration of hydrogen sulfide in water is highly dependent on pH, oxygen, and salt content [21±24]. Effects of hydrogen sulfide were tested in the open test vials of the Lumistrox test system. In contrast to the Lumistrox test, closed vials without headspace were used in the Daphnia and algae test. To control for the oxidation rate of H₂S in the closed test vials, a different set of experiments was used. The disappearance, i.e., volatilization and oxidation of H₂S, in undisturbed test vials was followed with an Analysensmesstechnik sensor (Fig. 2). In this way, the loss/oxidation rate of hydrogen sulfide in the tests over a certain testing time could be measured. The mean concentration of hydrogen sulfide over the whole test duration then could be calculated using the formula from the Organization for Economic Cooperation and Development [25] (http://www.olis.oecd.org/olis/2000doc.nsf/LinkTo/env-jm-mono[2000])

\[
\text{mean concentration} = \text{anti log} \left[ \frac{1}{2(t_n - t_1)} \sum_{i=1}^{n-1} \left( \log(\text{conc}n_i) + \log(\text{conc}n_{i+1}) \right) \right] \\
\times (t_{n+1} - t_1)
\]

where \( t_1 = \text{initial time} < t_2 < \cdots < t_n = \text{final time} \) and \( \text{conc}n_1 = \text{initial concentration} \), \( \text{conc}n_2, \ldots, \text{conc}n_n = \text{final concentration} \).

The analysis of hydrogen sulfide concentration in the ACES-buffered solutions were completed at 20°C with a 55 to 60% oxygen content. Concentration of hydrogen sulfide was measured regularly at different time points over 4 h. The mean concentration over the whole test duration of hydrogen sulfide in the three biotests with V. fischeri, D. magna, and S. vacuolatus test system then was calculated using Equation 1. The nominal concentrations in the tests then were corrected with the calculated mean concentrations. The nominal data sets and the calculated curve fits of both the nominal and corrected data were plotted in Figures 3 through 5.

Luminescent bacteria test

The Lumistrox tests were performed analogue to International Standard Organization 11348–1 using freshly prepared bacteria. The luminescent bacteria (V. fischeri, NRRL B-11177) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The bacteria were added directly to the buffered hydrogen sulfide testing solutions, and the luminescence after incubation times of 15 and 30 min was measured using a Lumistrox 400 luminometer (B. Lange, Düsseldorf, Germany). Tests with \( K \)-values below 0.7 and above 1.5 were defined as invalid. For each H₂S concentration tested, 10 parallel Lumistrox test vials were filled and analyzed. After the 30-min incubation time, the test solutions with the bacteria were
of using the M4 medium, the culture medium (Aachen Daphnia medium, ADaM) was prepared according to Klüttgen et al. [19] by mixing double distilled water with three 10-fold concentrated salt stock solutions and marine salt (Wiegand, Krefeld, Germany) reaching the specified end concentrations of ions (CaCl₂ × 2H₂O 1.84 mM, NaHCO₃ 0.66 mM, SeO₂ 0.013 μM, marine salt 0.33 g·L⁻¹). The performance of the Daphnia culture met the validity criteria for the test specified in the standard method [26]. Immobilized neonates after 24 and 48 h compared to controls served as effect parameter of toxicity. Modifications adapted the standard test system to the analysis of highly volatile and lipophilic substances. The test, therefore, was conducted in 15-ml gas-tight and closed Pyrex glass test tubes with Teflon sealings in the cap (Normag Labor & Prozesstechnik, Ilmenau, Germany). Double concentrated ADaM medium (7.5 ml) either was diluted with 7.3-ml pH-adjusted buffer (pH control) or buffered H₂S test solution. Neonates (aged 24–48 h) in 0.2 ml ADaM were added, and the immobilization of the neonates was examined after 24 and 48 h. For each concentration and control, 20 animals in four parallel pyrex vials (5 neonates each) were used. Oxygen concentration, pH, and hydrogen sulfide concentration were recorded at 0 and 48 h.

**Chronic algae toxicity test**

The unicellular green algae *S. vacuolatus* (formerly *Chlorella fusca var. vacuolata*) Shih et Krauss, strain 211–15, culture collection Pringsheim (SAG Göttingen, Germany) was used for testing. The procedure for conducting the assays followed the protocol described by Altenburger et al. [27]. The parameter of toxicity was the inhibition of the cellular reproduction during a one-generation cycle lasting 24 h, according to the aforementioned protocol. For counting of algae cells, a Casy®1 Counter (Scharfe System, Reutlingen, Germany) was used. The inhibition of cell reproduction was calculated by normalizing the data to the results of the control cultures. Adaptations to the above protocol were made in terms of sample application. Ten-milliliter Pyrex glass vials were filled with 7.2 ml of the ACES-buffered H₂S solution in the respective concentrations and diluted with 0.8 ml (10× concentrated) Grimme-Boardman medium [20] (pH 6.3), including the algae in a final starting concentration of 7.5 × 10⁴ cells/ml. To ensure carbon supply (1.5 mM final concentration), 20 μl of 0.6 M NaHCO₃ was added and the vials with Teflon seals were closed tightly.

In all three tests the EC50 values were estimated using nonlinear regression models based on a logistic distribution of the responses (SigmaPlot 8, SPSS Science, Erkrath, Germany).

**Modeling SSDs database**

The online Aquatic Toxicity Information Retrieval, U.S. Environmental Protection Agency Ecotox database and Pesticide Action Network from October 2004 was used to retrieve toxicity data for aquatic organisms exposed to hydrogen sulfide. The data used were based solely on acute tests (24 – 96 h) with freshwater and marine crustaceans as test organisms and immobility and mortality as test endpoints (EC50 and LC50).

Concentrations of hydrogen sulfide were either taken directly from the literature or, in case the pH was delineated, calculated from denoted total sulfide concentration by the use of the Henderson-Hasselbalch equation using a pKₐ of 7.01 [16]. If more than one toxicity value was available for the
same species, arithmetic means of the effect concentration values in each study were calculated for each species where possible. This way, each study was weighted in a similar way. To see if differences in sensitivity between freshwater and marine crustaceans exist [28], the SSD of both groups was calculated separately.

Empirical SSDs were calculated according to [29]. Toxicity data were plotted cumulatively. To obtain cumulative frequency distributions, percentages were calculated from the formula \((100 \cdot n) / (N + 1)\), where \(n\) is the rank number of the data point and \(N\) the total number of points in the dataset.

**RESULTS**

**Buffer selection**

To ensure a minimum impact of buffer salts on the test organisms, different Good et al. [30] buffers were tested in parallel to standard Tris and phosphate buffers. Additionally, the concentration of the buffers was kept below 50 mM. The concentration of hydrogen sulfide in oxygen-deficient solutions is mainly pH-dependent [6,21]; the pH of the test solutions was adjusted to a pH between 6.2 and 6.5 to reach maximum possible hydrogen sulfide concentrations, combined with a minimum pH stress to the test organisms. Phosphate and Tris buffers did not show satisfying pH stability. Additional tests using either different MOPS buffers (pH 7.2 and 6.7) or one ACES buffer (pH 6.9) in the luminescent bacteria test gave satisfying results with the ACES buffer only. Because of dilution of the H\(_2\)S generator solution with the respective buffers (at a ratio of 1:2), only the ACES buffer could be used to synthesize H\(_2\)S solutions with a concentration above 0.117 mM. The pH of the other two buffers, with a maximal molarity of 40 mM, could not be stabilized above 6.2 (data not shown). Because the minimum pH in the luminescent bacteria test is specified to be at least pH 6.0 ([31]; http://www.iso.ch/iso/en/stdsdevelopment/techprog/workprog/TechnicalProgrammeSCDetailPage. TechnicalProgrammeSCDetail?printable=true&COMMID=3729), the MOPS, phosphate, and Tris buffers were not used further. To minimize possible imprecisions, and for comparison between the biotests, the same buffer (ACES, pH 6.9, 40 mM, synthesized and used at 20°C except at the luminescent bacteria assay with 15°C) was used for the following experiments in all biotests.

**Loss rate of hydrogen sulfide**

The data in Figure 2 show a similar loss rate of hydrogen sulfide in the test solutions, independent of the concentration at the start of the experiment. The mean loss rate of the tested hydrogen sulfide solutions (0.015–0.176 mM) was approximately 13% in the first 30 min (Table 1). In solutions with higher concentrations between 0.088 and 0.205 mM, the decrease in the first 30 min followed an exponential curve decay. This was followed by a more linear decay rate up to the end of the measuring time of 240 min. In contrast, the decay rate in hydrogen sulfide solutions with concentrations between 0.015 and 0.044 mM did not show this exponential curve progression.

**Effect of hydrogen sulfide on luminescent bacteria**

Toxic effects of H\(_2\)S in terms of luminescence inhibition significantly increased from nominal concentrations above 0.117 mM (Fig. 3). Highest inhibition (70%) was accomplished with a concentration of 0.302 mM H\(_2\)S. A sigmoidal

<table>
<thead>
<tr>
<th>Start concentration (mM)</th>
<th>Assumed test duration</th>
<th>Calculated geometric mean (mM)</th>
<th>Calculated mean loss (%) compared to start concentration</th>
<th>Overall mean loss after 30 min and 48 h</th>
<th>Curve parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.015</td>
<td>30 min</td>
<td>0.020</td>
<td>13.7</td>
<td>13% (1.13% ± 0.43%)</td>
<td>(c = 0.36)</td>
</tr>
<tr>
<td>0.088</td>
<td>30 min</td>
<td>0.057</td>
<td>29.1</td>
<td>39% (11.84% ± 4.83%)</td>
<td>(c = 0.77)</td>
</tr>
<tr>
<td>0.176</td>
<td>30 min</td>
<td>0.107</td>
<td>58.8</td>
<td>43% (14.76% ± 6.24%)</td>
<td>(c = 1.17)</td>
</tr>
<tr>
<td>0.117</td>
<td>24 h</td>
<td>0.114</td>
<td>58.8</td>
<td>43% (14.76% ± 6.24%)</td>
<td>(c = 1.27)</td>
</tr>
<tr>
<td>0.182</td>
<td>48 h</td>
<td>0.138</td>
<td>45.6</td>
<td>43% (14.76% ± 6.24%)</td>
<td>(c = 1.58)</td>
</tr>
</tbody>
</table>
Table 2. Test parameters controlled in the biotests after the respective test times (pH, oxygen saturation °C, and hydrogen sulfide [H₂S] concentration) including calculated loss and resulting calculated mean test concentrations [25] of H₂S in the tests. Effective concentration estimated using functions provided to inhibit luminescence, immobilization or algal reproduction by 50% (EC50) (a: Maximal effect = 100%, x₀; xₐ in sigmoidal formula; b: Slope parameter in sigmoidal formula)

<table>
<thead>
<tr>
<th>Parameter at end of test</th>
<th>Vibrio fischeri (30 min)</th>
<th>Daphnia magna (48 h)</th>
<th>Scenedesmus vacuolatus (24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH range in test</td>
<td>6.2–6.5</td>
<td>6.4–6.5</td>
<td>6.5–6.6</td>
</tr>
<tr>
<td>O₂ saturation (%)</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Test temp. (°C)</td>
<td>15</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>EC50 [mM]</td>
<td>0.276 (real concn.)</td>
<td>0.0085 (nominal)</td>
<td>0.141 (nominal)</td>
</tr>
<tr>
<td>EC50 corrected with mean loss (see Table 1)</td>
<td>—</td>
<td>0.0036</td>
<td>0.055</td>
</tr>
<tr>
<td>Curve fit</td>
<td>Sigmoidal</td>
<td>Sigmoidal</td>
<td>Sigmoidal</td>
</tr>
<tr>
<td>Curve parameters</td>
<td>a: 100.0000</td>
<td>a: 100</td>
<td>a: 100</td>
</tr>
<tr>
<td></td>
<td>b: 0.0786</td>
<td>b: 0.0008</td>
<td>b: 0.0015</td>
</tr>
<tr>
<td>x₀;</td>
<td>0.2764</td>
<td>0.0036</td>
<td>0.055</td>
</tr>
</tbody>
</table>

*Dependent on generated concentration of H₂S solution. Sigmoidal: y = a/[1 + e^{-(x-x₀)/b}].

Effect of hydrogen sulfide on S. vacuolatus reproduction rate

Hydrogen sulfide concentrations between 0.029 and 0.264 mM were tested in the reproduction inhibition assay. Parallel gaslight test vials were used for each concentration analyzed and compared to the algae growth in the respective control vials. Data are depicted in Figure 5. Data fit was calculated using a sigmoidal function. A nominal EC50 of 0.141 mM was estimated using the curve parameters depicted in Table 2. The algae showed a high inhibition of reproduction with concentrations of hydrogen sulfide above 0.147 mM. Because the dose-response curve is very steep (with a ratio between the EC75–EC50 of 1.02), several experiments were performed in the range between 0.088 and 0.176 mM. With the loss of 39%, the EC50 would decrease from nominal 0.141 mM to calculated 0.055 mM.

Species-sensitivity distribution

Data availability varied considerably for the marine/freshwater crustaceans and for phytoplankton.

Overall, 10 geni with 55 datasets were used for the calculation of a SSD of freshwater crustaceans, from which 21 datasets were from studies with Gammarus pseudolimneaus only. In contrast, 11 geni with 45 datasets were used for the SSD calculation for marine crustaceans, where the number of datasets per genus is distributed more equally. Chronic effect parameter inhibition of reproduction for algae in terms of EC50 data were very rare. Datasets for only two Chlorophyceae, one Euglenophyceae, and one Cryptophyceae were available. These datasets are displayed as phytoplankton in Figure 6.

The reported datasets for EC50 covered a range over 3.5 orders of magnitude from 0.293 μM to 1.174 mM H₂S. No systematic differences in terms of sensitivity to hydrogen sulfide could be seen between the freshwater and marine crustaceans. More obvious is the fact that the majority of the organisms are sensitive to hydrogen sulfide in a range between 2.93 and 59 μM. Striking was the very low sensitivity of the marine crustacean Carcinus maenas, and that three out of four phytoplankton species are as insensitive to hydrogen sulfide as the tested luminescent bacteria Vibrio fischeri. The comparison of the generated EC50 of this study to the literature datasets showed that the water flea D. magna can be defined as com-

Function with the curve parameters $a = 100$, $b = 2.6805$, $x₀ = EC50 = 0.276$ mM (Table 1) was used to evaluate the concentration-response relationship and the EC50. The H₂S generator has an upper limit of synthesizing 0.734 mM H₂S solution and, thus, a 1:2 dilution with the respective buffer will generate maximal concentrations of 0.367 mM; therefore higher concentrations under the described conditions were not examined.

The pH did not decrease below 6.2 (Table 2). Oxygen concentration always was above 50% saturation (Table 2). Because H₂S was detectable after a 30-min testing time, the solutions of the Lumistox vials were pooled for analysis, and the nominal concentrations, therefore, were corrected to real concentrations. Using the calculated loss rates (Table 1), an overall average loss rate of 13% in the first 30 min would have meant a nominal starting concentration of 0.298 mM. This is very close to the actual programmed concentration of 0.302 mM in the hydrogen sulfide generator.

Effect of hydrogen sulfide on D. magna immobilization

Daphnia neonates were 1.5 orders of magnitude more sensitive to hydrogen sulfide than the luminescent bacteria. Concentrations from 0.0021 up to 0.026 mM were tested in the closed, gaslight Pyrex vials (Fig. 4). The highest immobilization rates were reached with concentrations above 5.87 μM. A sigmoidal function (Table 2) was used to calculate the (nominal) data fit with the curve parameters: $a = 100.1973$, $b = 0.0644$, $x₀ = EC50 = 8.5$ μM. The immobilization rate increased with time; that is, immobilization rates at the end of the 48-h incubation were 10 to 20% higher than after 24 h of incubation. As in the luminescent bacteria assay, test solutions were pooled for oxygen analysis content and measurement of final hydrogen sulfide concentration. The H₂S was detected only with concentrations between 1.76 and 0.29 μM, irrespective of the starting concentration. This is at the very low end of sensor sensitivity. Oxygen saturation always was above 50% after 48 h (Table 2).

Using the average calculated loss rates from Table 1, the geometric mean concentrations of hydrogen sulfide in the Daphnia tests (48 h) significantly decreased to 57% of the nominal starting concentration. With the loss of 43%, the EC50 would decrease from nominal 8.5 μM to calculated 3.6 μM based on analytical concentrations.
Hydrogen sulfide effects on biotest organisms

![Species-sensitivity distribution of freshwater () and marine (▲) crustaceans and of different phytoplankton species (●).](image)

**DISCUSSION**

The objective of this research was to establish dose-response relationships for hydrogen sulfide (H$_2$S) of the three aquatic biotest organisms *V. fischeri*, *D. magna*, and *S. vacuolatus*. An H$_2$S generator was used to synthesize defined H$_2$S concentrations, circumventing many of the problems usually occurring with the classical synthesis of H$_2$S. The loss rate of the volatile H$_2$S in open and closed test vials was analyzed to correct for the assumed losses in the used biotest systems.

In literature, the toxicity of H$_2$S mainly was analyzed with different marine and freshwater sediment-dwelling organisms. The idea behind many tests is the fact that H$_2$S often is a very prominent factor in sediments, with a well-known toxicity. As a consequence, most authors tested several species from different geni under the aspect of adaptation mechanisms or sensitivity. Organisms tested in-
an EC50 of 0.126 mM (0.085–0.185 mM of 95% confidence interval) for Na2S with a 15-min incubation time. However, here only nominal concentrations were used. The calculation of the actual H2S concentration in the 15-min incubation time will give an approximate H2S concentration of 0.064 mM at pH 7 that is similar to the data of Postma et al. [3]. Thus, all literature cited show a four times higher toxicity of H2S to the bacterium V. fischeri than the results presented here. Reasons for the differences can be based on either the assumption that the synthesis in the cited literature data were different or that the authors did not measure real concentrations at the end of the tests and, therefore, were not able to correct their data.

The data presented here showed that the EC50 (8.51 μM) of D. magna water fleas using nominal concentrations is very comparable to the sensitivities of other freshwater and marine crustaceans. The D. magna neonates tested here were among the most sensitive group of animals (Fig. 6), with the mayfly Baetis vagans and the gammarid Gammarus pseudolimnaeus as the most sensitive organism. van Leeuwen et al. [39] calculated the LC50 (using Na2S) for the D. magna 48-h test to be 61.6 μM. The test vessels were sealed and the test solutions were prepared fresh and renewed daily so that the test conditions remained well defined. Because the pH in the test was close to the pKs-value of H2S (7.01) [16], the corrected EC50 of H2S would be 32 μM, that is, about four times higher than the EC50 measured in this work. An explanation would be the fact that van Leeuwen et al. [39] renewed the test media after 24 h. By implicating the calculated mean loss (39% after 24 h see Table 1), a mean concentration of 19.6 μM could be assumed. Because the loss rates of low concentrated hydrogen sulfide solutions are thought to be much higher (Table 1), one could assume somewhat higher losses in the test so that the actual EC50 might decrease even below the modeled 19.6 μM. Using the same data for the calculated loss rates, the EC50 of our biotest would be adjusted to 3.6 μM instead of the nominal EC50 of 8.51 μM. The difference between the two experiments of van Leeuwen et al. [39] and our tests, therefore, could decrease to a factor between two to four. Because the authors did not write whether or not quantification of H2S was performed, we assume that the differences between data of van Leeuwen et al. [39] and our results are based on the differences in synthesis and control of the hydrogen sulfide solution and its concentrations.

Another interesting point is that the EC50 of 3.6 μM with the D. magna neonates is a very low concentration. As shown in Figure 2 and Table 1, no hydrogen sulfide would be left in the closed vials after 10 h if the starting concentration was below 22 μM. However, although no hydrogen sulfide remains in solution after a 10-h test time, the animals still are affected for at least another 48 h. Consequently, future studies could include recovery experiments to analyze if and how long the effect of immobilization by hydrogen sulfide is reversible.

van Leeuwen et al. [39] calculated an EC50 of 75 mg L−1 Na2S (i.e., 1.03 mM H2S at pH 7) in an algae test. The data presented here are based on a nominal EC50 of 0.1408 mM in the chronic algae assay, which would decrease to a calculated EC50 of 0.055 mM using the loss rate of 39% after 24 h (Table 1). van Leeuwen et al. used open test vials, and the duration of the test was 96 h. In contrast, the test conditions here included sealed vials and a test duration of only 24 h. This might explain the higher EC50 by van Leeuwen et al. [39], because H2S might have been volatized and oxidized during the biotest, so that much higher concentrations of the Na2S had to be used to get any effect.

Comparing the sensitivity of the algae tested, the two Scenedesmus species are more sensitive than the other three phytoplankton species (Fig. 6), but are among the lesser sensitive organisms compared to the analyzed crustaceans. However, because the number of algae or other phytoplankton tested in a reproduction assay is very low, future studies should include more chronic assays instead of commonly used acute tests based on the measurement of photosynthesis inhibition [40]. This enables a better comparison between microalgae and other organisms.

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REFERENCES


