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# **Supplementary Guidance for Investigating Acute Lethality of Pulp and Paper Mill Effluents due to Ammonia**

Method Development and Applications Section  
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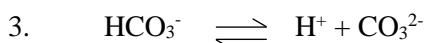
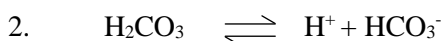
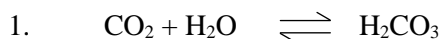
# Supplementary Guidance for Investigating Acute Lethality of Pulp and Paper Mill Effluents due to Ammonia

## 1.0 Introduction

This appendix provides background information and supplementary guidance on procedures used to identify un-ionized ammonia as the cause of acute lethality in pulp and paper effluent.

## 2.0 Background

The role of pH as a modifying factor of toxicity has been well established (Sprague, 1995). Due to the pH sensitivity of many toxicants, even small changes in pH can have a marked effect on toxicity. The pH of natural waters is greatly influenced by carbon dioxide (CO<sub>2</sub>). Although CO<sub>2</sub> is not acidic itself, it can react with water to make acidic solutions by forming carbonic acid (H<sub>2</sub>CO<sub>3</sub>), which can then be dissociated to release hydrogen ions according to the following equations (Weiner, 2000):



At equilibrium, bicarbonate and carbonate ions dissociate and hydroxyl ions (OH<sup>-</sup>) can be formed by the hydrolysis of carbonic acid. Un-ionized CO<sub>2</sub> predominates below pH 6, while HCO<sub>3</sub><sup>-</sup> predominates between pH 6 and 10. Carbonate (CO<sub>3</sub><sup>2-</sup>) dominates above pH 10 (Rand, 1995). The above equations (representing a simplified version of the carbonate buffer system) can influence the pH of virtually any circum-neutral aqueous solution.

In many effluent samples, the CO<sub>2</sub> content may be artificially elevated as a result of high biological activity or a result of effluent acidification prior to discharge (Mount and Mount, 1992). Consequently, aeration (which is a test method requirement for both the control and test solutions) of pulp and paper effluents during acute lethality testing may cause the pH to rise from the equilibration of CO<sub>2</sub> partial pressure in the effluent with that in the atmosphere. The loss of CO<sub>2</sub> due to aeration causes a shift in the carbonate buffering system of an effluent, and this leads to the rise in pH (Mount and Mount, 1992; Wisconsin Department of Natural Resources, 2005, 2004). Any change in pulp and paper effluent pH during an acute lethality test may affect mortality if the toxicity of the substance responsible is pH-dependent. Ammonia, which could be of concern in pulp and paper effluent, would be one such example of a pH-dependent toxicant.

Ammonia toxicity is attributable to the free or un-ionized (NH<sub>3</sub>-N) form as opposed to the ionized species. The relative concentration of un-ionized ammonia increases with increases in pH and water temperature. The percentage of un-ionized ammonia in aqueous total ammonia solutions as a function of pH and temperature (based on the acceptable range of temperatures for the test method; i.e., 14 to 16°C) are provided in Table 1. To calculate the concentration of un-ionized ammonia using the values presented in Table 1, the measured total ammonia concentration is multiplied by the corresponding value for the appropriate pH and temperature of the solution. For example, for a total ammonia concentration of 10 mg/L, the corresponding

concentration of un-ionized ammonia at pH 8.5 and a temperature of 15°C, is 0.80 mg/L (i.e., 10 x 8/100).

**Table 1. Multiplication factors (%) for calculating un-ionized ammonia at 14–16 °C and pH 6 – 9.5 \***

Temperature (°C)	pH							
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
14	0.025	0.080	0.25	0.80	2.5	7.4	20	45
15	0.027	0.087	0.27	0.86	2.7	8.0	22	46
16	0.030	0.093	0.29	0.93	2.9	8.5	23	48

\*from Emerson *et al.*, 1975

Depending on the initial pH (pH i)<sup>1</sup> of the full-strength pulp and paper effluent and the magnitude of the upwards pH drift during testing (caused by a loss of carbonic acid, driving the reaction towards the formation of CO<sub>3</sub><sup>2-</sup>; see equation 3 above), concentrations of un-ionized ammonia that were below lethal levels at test initiation, could increase sufficiently during testing to cause rainbow trout mortality by test completion.

Thurston *et al.* (1981a, b) showed that the toxicity of un-ionized ammonia to rainbow trout varied with pH and alkalinity. Over the range of pH (6.5 to 9.0) and alkalinity (75 to 196 mg/L as CaCO<sub>3</sub>) tested, un-ionized ammonia toxicity was inversely proportional to both of these parameters. That is, while more un-ionized ammonia is formed at higher pH, the same concentration of un-ionized ammonia is more toxic at lower pH and alkalinity. For example, concentrations of un-ionized ammonia as low as 0.13 mg/L (Thurston *et al.*, 1981b) have caused acute toxicity to rainbow trout in waters with low pH (6.4 to 6.7) and alkalinity (62 to 86 mg/L as CaCO<sub>3</sub>). However, this value is higher (0.66 mg/L) with increasing pH (8.2 to 8.8) and alkalinity (~ 190 mg/L as CaCO<sub>3</sub>). Kovacs *et al.* (2004) reported LC50s for un-ionized ammonia ranging from 0.4 to 0.6 mg/L.

### 3.0 Conditions for Use of pH Stabilization Procedures – Other Effluents and Jurisdictions

In 2008, pH stabilization methods were published by Environment and Climate Change Canada for use with municipal wastewater effluents (EPS 1/RM/50; EC 2008a). Several jurisdictions in the United States (e.g., Washington State Department of Ecology, 2001; Colorado Department of Public Health and Environment, 1998; Wisconsin Department of Natural Resources, 2004, 2005) also allow for control of pH during toxicity testing in order to reduce the occurrence of ammonia toxicity due to pH drift. In addition, the US EPA (2002 a,b) acute and chronic test methods provide guidance on the effect of pH and temperature on ammonia toxicity, as well as some flexibility in allowing for pH control during toxicity testing, provided evidence is gathered to verify that the source of mortality is caused by pH drift.

<sup>1</sup> pH i refers to the pH as measured on composite 100% sample at 15 ± 1°C before any aeration of the test solution at the lab.

In the aforementioned US jurisdictions where pH stabilization is allowed, adjustment of pH during the toxicity tests is not conducted arbitrarily, but is based on a definitive set of criteria (depending on the specific jurisdiction). Examples of criteria, which must be met prior to conducting pH stabilized toxicity tests include:

- demonstration that toxicity has been observed due to pH drift,
- parallel testing of pH stabilized and un-stabilized samples at all times,
- weight of evidence that establishes ammonia as the cause of toxicity (e.g., using Toxicity Identification Evaluation (TIE) methods),
- demonstration that a minimum ammonia concentration is measured where acute lethality might be observed, and
- demonstration that measured ammonia concentrations are below maximum chemical limits set by local or regional authorities.

In this method, in order to understand if rainbow trout mortality is a result of increases in un-ionized ammonia caused by pH drift during the 96-h exposure, it is important that toxicity tests with pulp and paper effluent samples meet four conditions:

1. Measurement of total ammonia at the start and end (minimum) of the pH stabilized and un-stabilized toxicity test with rainbow trout; more frequent (as much as daily) may also be taken.

In biological treatment systems, nutrients (including ammonia) released from anaerobic breakdown of settled sludge/solids is referred to as benthic feedback (FPAC, 2008). If this occurs, there is a potential for ammonia to be released during a toxicity test, resulting in a gradual increase in total ammonia during the 96-h exposure. This increase can also occur during transportation or holding of the sample prior to testing. Nutrients can also be consumed by bacteria (resulting in a net decrease in ammonia in pulp and paper effluent samples submitted for toxicity testing).

For these reasons, total ammonia (in the 100% sample) must be measured at test initiation (0-h) and test completion (96-h), and at any time during the test when >50% mortality is observed. Ideally, total ammonia and corresponding pH would be measured daily (i.e., every 24-h monitoring period) in both the pH stabilized and unstabilized test.

Total ammonia measurement, in combination with pH and temperature are then used to calculate un-ionized ammonia concentrations, and assess if concentrations were sufficiently elevated to explain the observed trout mortality.

2. The pH stabilization procedure must only be used when the un-ionized ammonia concentration present in the 100% effluent sample does not equal or exceed 1.25 mg/L at 15°C or when the total ammonia concentration does not equal or exceed the maximum total ammonia concentration (y) in mg/L determined using the following formula and the initial pH of the effluent sample at 15°C:

$$y = 1.25 \times (10^{(9.564 - \text{pH})} + 1)$$

In December 2004, a Guideline for the Release of Ammonia Dissolved in Water Found in Wastewater Effluents was published as a notice under the Canadian Environmental Protection Act, 1999 (CEPA 1999). As part of this CEPA 1999 notice, a revised formula to predict ammonia toxicity ( $y = 306132466.34 \times (2.7183^{(-2.0437 \times \text{pH})})$ ) was published (Environment Canada, 2004a). This formula was based on the lower 95% confidence limit of the final regression formula generated by Pacific Environmental Science Centre (i.e.,  $y = 326597301 \times e^{(-2.0384 \times \text{pH})}$ , Figure 1).

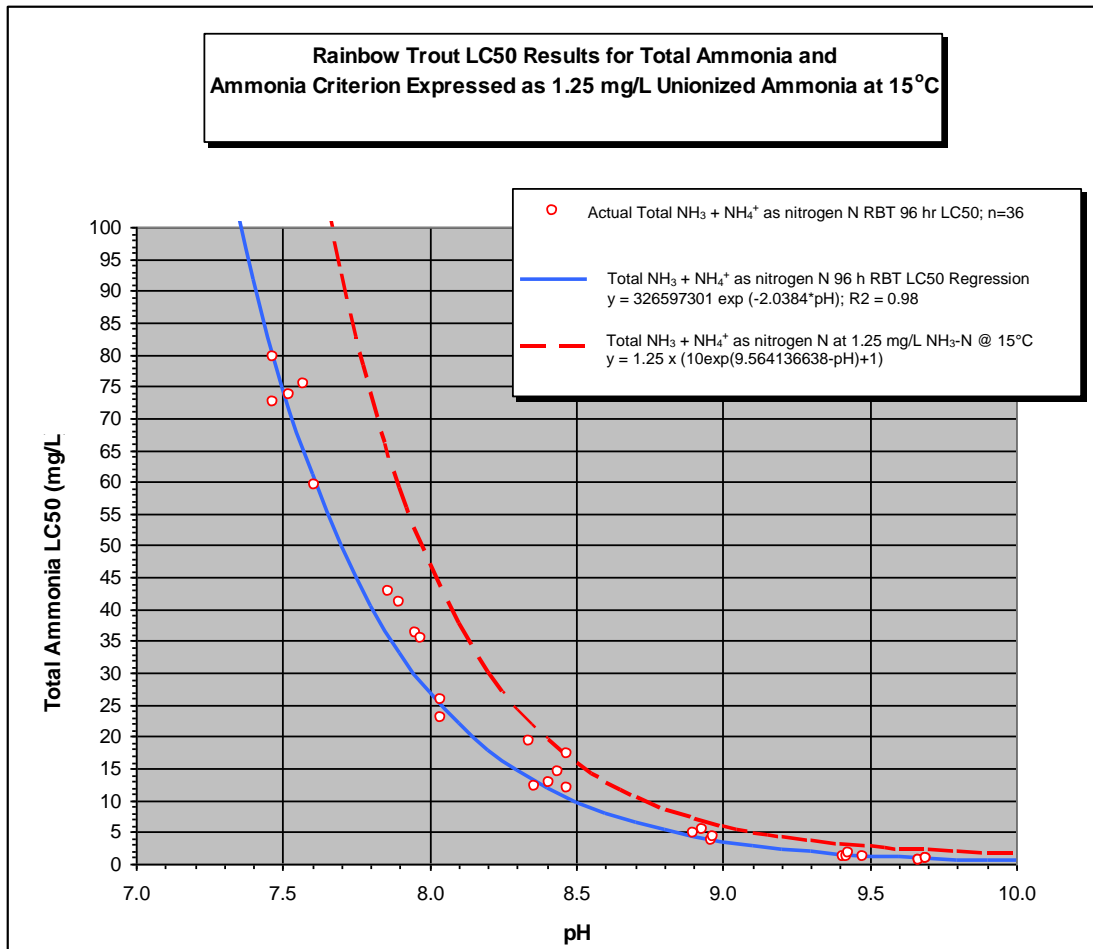


Figure 1. Total ammonia 96-h rainbow trout LC50 results showing regression curve versus total ammonia expressed as 1.25 mg/L un-ionized ammonia at 15°C (Environment Canada, 2004b).

These maximum values for ammonia are set to pre-screen effluents that would result in rainbow trout mortality regardless of the pH drift observed during the acute lethality test. In other words, pH stabilization techniques are not appropriate if the ammonia concentration is already sufficiently high to cause rainbow trout mortality at the start of the acute lethality test. If this maximum un-ionized ammonia value is exceeded, it clearly

identifies that an effluent is not of a quality where the pH drift phenomenon would be a consideration (i.e., ammonia is already at an acutely lethal concentration prior to testing).

For example, an effluent sample at pH 8.2 with a total ammonia concentration of 45 mg/L, would not be eligible for pH stabilization, since the corresponding un-ionized ammonia concentration of 1.9 mg/L would be >1.25 mg/L. This concentration of un-ionized ammonia would be acutely lethal to rainbow trout regardless of any increase in the un-ionized form caused by pH drift during the test. In comparison, pH stabilization would be recommended in an effluent sample at pH 8.4 and a total ammonia concentration of 13 mg/L, since the corresponding un-ionized ammonia concentration of 0.84 mg/L is less than 1.25 mg/L.

3. The pH stabilization procedure is only conducted on 100% effluent (single concentration) because the method was only validated for this test design (i.e., not multi-concentration tests to determine the LC50).

The reference method for pH stabilization of pulp and paper effluents is only permitted for use with the 100% full strength sample (i.e., the Single Concentration test in Section 5 of EPS 1/RM/13). However, multiple concentration (LC50) pH stabilized tests can be conducted as part of investigation tests and may provide additional information on the possible presence of toxicants other than un-ionized ammonia (e.g., data from LC50 tests could be useful in the event toxicity is observed in diluted effluent concentrations where calculated un-ionized ammonia concentrations are below thresholds shown to be acutely lethal to rainbow trout).

4. Parallel testing with Reference Method EPS 1/RM/13

Parallel testing is required on all samples to consistently demonstrate the presence of ammonia toxicity and pH drift, and to confirm that other pH sensitive toxicants are not present at acutely lethal concentrations. If the sample is acutely lethal using the standard reference method this documents whether there was pH drift and sufficient unionized ammonia to cause toxicity. This provides the rationale/evidence that the add-on pH stabilization procedure is appropriate to use.

#### **4.0 Investigation Procedures for Identification of Un-ionized Ammonia as the Cause of Rainbow trout Mortality**

Ammonia is a toxicant that can act independently of other toxicants, and other compounds that are sensitive to pH change may also be present in an effluent sample (Wisconsin Department of Natural Resources, 2005; US EPA, 1999a). Therefore, an investigation will be critical to demonstrate that increases in the pH of pulp and paper effluent during testing result in acute lethality exclusively due to ammonia.

No single test can be used to confirm the presence of ammonia toxicity. Rather, a combination of approaches and treatments are used to provide “weight-of-evidence” supporting ammonia as the substance responsible for acute lethality. Tests could include:

1. parallel tests with and without pH stabilization (as required by this test method);
2. species sensitivity comparisons using *Daphnia magna*;
3. zeolite treatment of the effluent, and
4. extended air-stripping of the effluent at high pH (i.e., pH 11).

If the above tests (combined with measured pH and ammonia concentrations) consistently indicated mortality due to ammonia, then it is unlikely that a hidden toxicant (e.g., metals, sulfide, resin and fatty acids) is present in the effluent, and observed mortality can be attributed to the presence of ammonia (US EPA, 1993b). In the event that the “weight-of-evidence” does not support ammonia as the primary cause of mortality, methods and treatments are available to investigate other toxicants (US EPA, 1991, 1993a, b, 1999a)

The approach and treatments for building a “weight-of-evidence” for ammonia as the primary cause of rainbow trout acute lethality are based on the US EPA (1991, 1993a, b) TIE procedures, as well as other guidance documents (ESG, 2002; Wisconsin Department of Natural Resources, 2005). A brief overview of the tests and treatments are provided in the following section. The US EPA TIE methods should be consulted for a more detailed description of the methodology.

#### **4.1 Parallel Testing**

Parallel tests with and without pH stabilization are required in this test method to demonstrate the presence of ammonia toxicity caused by the pH upward drift during acute lethality testing. In the test without pH stabilization, the sample is tested according to the Environment Canada EPS 1/RM/13 (2000a) test method, and the pH is allowed to drift. In the pH stabilized sample, the pH is controlled according to procedures described herein. In addition to the standard lab control, pH stabilized controls are also used to ensure that the treatment itself does not cause mortality. There are several advantages to using CO<sub>2</sub> direct addition with the pH Controller techniques and these have been described in various research papers and government documents (Elphick *et al.*, 2005; Wisconsin Department of Natural Resources, 2004, 2005; Colorado Department of Public Health and Environment, 1998; Mount and Mount, 1992; US EPA, 1991) and include:

- CO<sub>2</sub> has minimal disturbance on the existing Environment and Climate Change Canada test conditions.
- CO<sub>2</sub> uses a natural buffer system and represents an on-going control of pH, rather than a temporary adjustment (as is with acid/base additions).
- acids and bases can disturb the effluent carbonate balance. Acids and bases need to be continuously added to maintain pH, since carbonate equilibrium is rapidly re-established after adjustment.
- toxic artifacts are generally avoided with CO<sub>2</sub>. Toxic artifacts could occur if sufficient acid or base were to be added such that the overall total dissolved solids of the effluent would be altered (e.g., chloride toxicity resulting from the addition of hydrochloric acid).



- the use of flow-through tests (onsite or in the lab) as a means to maintain pH is expensive.
- the addition of buffers (e.g., Bis-Tris buffer, MOPS, POPSO) could alter effluent chemistry and contribute to the overall toxicity as well.

At test completion, results from the unstabilized and stabilized tests are compared:

- if rainbow trout mortality occurs in the un-stabilized, but not in the pH stabilized test, increased levels of un-ionized ammonia due to pH drift or pH-influenced toxicity is likely; additional investigative testing is still required to demonstrate un-ionized ammonia was the only cause of toxicity,
- if rainbow trout survive in both the pH stabilized and un-stabilized tests, the results bring into question the need to run parallel testing; it is also possible that that ammonia toxicity may be transient (assuming historical tests have shown pH stabilization tests have been non acutely lethal to rainbow trout),
- if mortality results are similar in both tests, then acute lethality is not due to un-ionized ammonia toxicity as a result of pH drift in the test, and
- if the pH stabilized test is acutely lethal to rainbow trout, but the uncontrolled pH test is not acutely lethal, then acute lethality could be due to the presence of other pH sensitive toxicants (e.g., metals, resin acids, hydrogen sulfide) in the effluent sample. For example:
  - resin acids exceeding 1 mg/L can be acutely lethal to rainbow trout, and behave the opposite of ammonia whereby resin acid toxicity decreases with increasing pH (Kovacs *et al.*, 2004)
  - copper solubility increases (and, therefore becomes more toxic) as pH decreases (i.e., from pH 8 to pH 7) (Miller and Mackay, 1979). Consequently, copper toxicity may not be observed in an effluent - where an upward pH drift is observed (i.e., the uncontrolled pH test). However, increased mortality due to copper may be observed when a relatively lower pH is maintained throughout the test (i.e., the pH stabilized test). Schubauer-Berigan *et al.* (1993) reported that acute lethality of copper and lead to a variety of test organisms (e.g., fathead minnows, *Ceriodaphnia dubia*, *Hyaella azteca*) was greatest at pH 6.3 and least at pH 8.3. Conversely, toxicity of cadmium, nickel and zinc was greatest at pH 8.3. Ammonia and metals were identified as the cause of pore water toxicity to *Ceriodaphnia dubia* (Schubauer-Berigan and Ankley, 1991). When evaluated at three pH levels (6.5, 7.5 and 8.5), toxicity was greatest at pH 6.5 (likely due increased metals bioavailability and toxicity at pHs lower than neutral) and pH 8.5 (likely due to increased un-ionized ammonia).

## 4.2 Species Sensitivity (comparisons to *Daphnia magna*)

Species sensitivity comparisons are useful as part of the “weight-of-evidence” to support ammonia as the cause of effluent toxicity (Wisconsin Department of Natural Resources, 2005, 2004; ESG, 2002). In the case of ammonia, rainbow trout are more sensitive than *Daphnia magna*. *Daphnia magna* is another commonly used test species, for which there is a standardized Environment Canada (2000b) Reference Method (EPS 1/RM/14).

As part of the “weight-of-evidence” approach, tests should be conducted on the same sample with both rainbow trout and *Daphnia magna* to demonstrate the relative species sensitivity. In cases where the effluent is acutely lethal to *Daphnia magna*, but non lethal (or significantly less toxic) to rainbow trout, ammonia might not be a likely cause of acute lethality. However, species sensitivity comparisons alone do not provide definitive confirmation that ammonia is the cause of acute lethality. For example, if the effluent exhibits comparable toxicity to both species, then multiple toxicants (including ammonia) might be present in the effluent. Therefore, this information must be combined with results from other treatments (e.g., pH stabilization test, zeolite, and ammonia stripping) and with measured ammonia concentrations that are known to cause acute lethality.

## 4.3 Zeolite Treatments

Zeolites can be used to remove ammonia from effluent samples. Zeolites are crystalline aluminosilicates, which exhibit high selectivity for ammonia, but can also remove some heavy metals (Sherman, 1978).

The general testing approach involves rinsing zeolite resin with reverse osmosis (RO), de-ionized or distilled water, followed by dilution water. A portion of dilution water (blank) should be collected for toxicity testing. The 100% effluent is passed through next and tested to determine toxicity of the 100% treated sample. Samples of effluent should be analyzed for ammonia before and after zeolite treatment. Ammonia removal efficiency can be affected by column packing, effluent pH, ammonia concentrations, and flow rate, etc., and some preliminary testing should be conducted to confirm treatment conditions will be effective for the effluent under investigation.

The presence of other toxicants might be suspected if ammonia is removed by zeolite (based on measured concentrations after treatment), and the zeolite-treated effluent is still acutely lethal. However, if the zeolite treated effluent is non-lethal, it cannot be concluded that ammonia is the only toxicant, since zeolite may remove other substances (i.e., metals) in addition to ammonia (US EPA, 1993a). Results from the zeolite treated effluent must be used in combination with other manipulations to confirm ammonia as the substance responsible for acute lethality.

In cases where zeolite effectively removed mortality, and reduced ammonia concentrations to below lethal levels, “spike-back” tests should be conducted to provide further evidence that ammonia was the main cause of acute lethality. If mortality is restored in the post-zeolite treated effluent by spiking with ammonia (at a concentration similar to the original effluent concentration), then it is likely there was sufficient ammonia in the sample to render the effluent acutely lethal (US EPA, 1991).

#### 4.4 Ammonia Stripping

Air-stripping of ammonia from an effluent sample takes advantage of the fact that relatively volatile un-ionized ammonia is present in solutions with pH greater than 9.3. Ammonia in water at pH 9.3 (the pKa of ammonia<sup>2</sup>) will exist as 50%  $\text{NH}_4^+$  and 50%  $\text{NH}_3$ . At one pH unit above the pKa, approximately 90% of the ammonia will be in the un-ionized form ( $\text{NH}_3$ ), and the remainder will be in the ionized form ( $\text{NH}_4^+$ ) (US EPA, 1991).

Air-stripping of ammonia requires stirring of the sample at high pH (>10) for an extended period (e.g., >1 hour; or until total ammonia concentrations are reduced to below acutely lethal levels) in a container with a large surface area to volume ratio. The sample is then readjusted to the initial pH of the effluent and tested for acute lethality. Caution is required when adjusting effluent pH as excessive acid and/or base addition can increase the total dissolved solids content of the effluent sample resulting in toxicity after treatment due to elevated ionic strength of the treated sample.

Ammonia will be strongly suspected as contributing to toxicity, if ammonia and mortality are both reduced after air-stripping. These results should be compared with the above treatments/tests, since other potentially toxic substances could precipitate (and not re-dissolve) after air-stripping.

In cases where air-stripping rendered the effluent non lethal, and reduced ammonia concentrations to below acutely lethal levels, “spike-back” tests should be conducted to provide further evidence that ammonia was the main cause of acute lethality. If the effluent is acutely lethal after being spiked with ammonia, then it is likely there was sufficient ammonia in the sample to render the effluent acutely lethal (US EPA, 1991).

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<sup>2</sup> pKa = - logKa, where Ka is the acid dissociation constant

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