RAPID TOXICITY ASSESSMENT USING *COLPODA STEINII*: TOXICITY OF IBUPROFEN, NAPROXEN, AND ACETAMINOPHEN

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Abstract

A rapid microscale bioassay using the ciliate *Colpoda steinii* was developed to determine growth inhibition by over-the-counter pain relievers ibuprofen (IBP), naproxen (NAP), and acetaminophen (APAP). Formulated over-the-counter (OTC) products and pharmaceutical grade material were analyzed. Experiments also examined copper (as Cu^{+2}) as a reference toxicant. Growth experiments of four replications of 4-5 test concentrations plus controls for 48 hr were analyzed by regression, ANOVA, and multiple comparisons to identify the EC_{20}, EC_{50}, NOEC, and LOEC for each toxicant. Results varied from a low NOEC of $10mgL^{-1}$ for ibuprofen to a high EC_{50} of $631mgL^{-1}$ for acetaminophen and compared favorably to other tests. *C. steinii* is suitable for rapid testing and hazard ranking for compounds such as PPCP's in water systems. The assays are cost-effective, reliable, and can assist in screening and understanding potential adverse effects of multiple toxicants.

INTRODUCTION

Microscale bioassays offer toxicological evaluations of effluents, leachates, sediments, and other environmental matrices that are rapid, cost-effective, and practical (Wells et al 1998). Tests organisms range from bioluminescent bacteria (*Vibrio fischeri*) such as Microtox® and Mutatox® to cyst-based kits for testing protozoa and rotiferes (eg. Protoxkit, Rotoxkit). Small-scale testing has an important role in evaluating new and existing chemicals (Wells et al 1998).

Organisms that form cysts and resting stages are especially valuable for developing rapid test systems. Cysts can be stored dry and used when needed. Rotifers have been utilized in aquatic toxicity bioassays since the early 1990's, because cysts can be dried and stored for years and then hatched at room temperature (25°C) in 24 hours by the addition of water (Snell and Janssen, 1996). Eliminating the need for long-term culture of test organisms saves time and decreases costs. Because all labs can start with a similar strain, in the same physiological condition, standardization and reproducibility of the cyst-based tests is increased (Snell and Janssen, 1996).

Ciliate excystment can occur after six weeks of desiccation and freezing (Müller et al 2009). Foissner (1993) stored *C. steinii* cysts in a vacuum for upwards of 7 years, suggesting cysts can be stored and utilized as needed. Cysts of some ciliates are available from the American Type Culture Collection. Rapid growth rate of many ciliates (doubling 3-4 times per day) and ease of culturing make them ideal for growth inhibition bioassays (Pratt et al 1997).

Pharmaceuticals and personal care products (PPCP's) are increasingly found in many water systems around the world. Over-the-counter pain medications, prescribed medications like antibiotics and antidepressants, and care products such as detergents and fragrances occur in aquatic ecosystems. Pain relieving and anti-inflammatory drugs are of particular concern due to their frequency of use. In the U.S., 70 million prescriptions for non-steroidal anti-inflammatory

drugs (NSAID's) are written each year. Prescription and OTC products account for 30 billion doses in the United States alone (Hwang et al., 2013). Globally, NSAID and other pain reliever use is increasing and raises questions of the fate and effects of PPCP's in aquatic ecosystems. Studies have reported influent concentrations of ibuprofen, naproxen, and acetaminophen into wastewater treatment plants (WWTP's) ranging from 838 to 492,340 ngL^{-1} (Petrie et al., 2015). Although removal efficiency through WWTP's is often high (>80%), pain relieving compounds make their way through wastewater systems to rivers and streams (Jelic et al 2011). Ranking the hazard of these and other chemicals in ways that are rapid, reliable, and inexpensive is important to begin to understand the impact of these PPCP's in the environment.

The purpose of our experiments was to develop a rapid bioassay using *C. steinii* and to determine the sensitivity and variability of response to PPCP's. *C. steinii* are ubiquitous, significant recyclers and remineralizers of organic material in both terrestrial and aquatic environments, have rapid population growth rates, and are relatively easy to culture and maintain (Gilron and Lynn 1998).

MATERIALS AND METHODS

Colpoda steinii (Maupas 1883) was isolated from rehydrated leaf litter collected near the Columbia River in Richland, WA. *C. steinii* is a small ciliate, 20-40 x 15-30 um commonly found in soil and enriched freshwaters (Foissner 1993). *C. steinii* were isolated from the sample and transferred to a 10% Sonneborn's *Paramecium* medium (SPM) for 2-7 days (Pratt et al 1997). Within 48-72 hours, 1mL of culture was removed and subcultured into 4mL of fresh medium for use in experiments. Old cultures containing cysts were filtered through 25µm pore size, 55mm filters (Whatman #42) and allowed to dry in Petri dishes. Once dried, the stored filters were used to start cultures for further experiments.

SPM was made by boiling 1.25g of dehydrated wheatgrass (cereal leaves) in 500mL of deionized water for 5 minutes with continuous stirring. The mixture was filtered to remove particulates, autoclaved, and the final volume was readjusted to 500mL with deionized water (Pratt et al 1997). Full-strength medium was refrigerated for up to 7 days and diluted to 10% as needed. Diluted medium was buffered with Na_2HPO_4 ($0.5gL^{-1}$), and pH was adjusted to 7.5-8.

Toxicity tests were conducted in 10 x 75mm disposable glass culture tubes at room temperature (19 – 23°C). Sterile medium was dispensed into culture tubes and amended with toxicant stock solutions made from over-the-counter or pharmaceutical grade material prepared in sterile 10% SPM (final volume 2ml). Approximately 200 cells from ciliate culture (~10 ul) were added from log phase cultures (48-72hrs), and 10µL of non-pathenogenic *E. coli* culture was added to each culture tube. If necessary, *E. coli* cultures were diluted (absorbance ~0.700 at 600nm) with nutrient broth prior to each experiment to control bacterial population size.

The test period was 48 hours ($\pm$ 2hrs). After 48 hours, subsamples were removed from each replicate culture tube and enumerated using a direct counting technique. Each replicate was mixed using a vortex mixer, stirred with a micropipettor, and a 10µL subsample was transferred to a clean microscope slide as 6 to 8 random drops. These drops were immediately scanned at low magnification (40 x) on a compound microscope to search for active cells. This was done twice for each replicate (total of 20µL) to estimate the population density. This procedure was repeated for each replicate; the average of subsample estimates for a given replicate was used in analyses.

For all tests, unreplicated preliminary range-finding tests were done to establish the probable toxic range for each tested compound. These tests used only pharamaceutical grade material. Subsequent testing involve preparation of fresh stock solutions prior to experiments.

All stock solutions were prepared no more than 72 hours in advance of each experiment and discarded after the experiments were completed. Stock solutions were refrigerated (4˚C) and were brought to room temperature (20°C) prior to use.

Ibuprofen (IBP), 4-isobutyl-alpha-methyl-phenylacetic acid, was used in two forms. Pharmacological grade material ((99%, CAS# 15687-27-1, LOT: C7520A) was acquired from Alfa Aesar, Ward Hill, MA. The OTC product was Advil® Liqui-Gels®, 200mg Liquid-Filled Capsules, distributed by Pfizer, Madison, NJ (LOT: R37020, EXP: 04/19).

IBP is a NSAID that interacts with cyclooxygenases (COX-1, COX-2), inhibiting inflammation and providing pain relief. Cyclooxygenases are intracellular enzymes which modify the inflammation response by that catalyzing the conversion of arachidonic acid into biologically active lipids called prostanoids (Hwang et al., 2013).

Naproxen (NAP), (S)-(+)-2-(6-Methoxy-2-naphthyl) propionic acid, was used in two forms. Pharmacological grade material (99%, CAS# 22204-53-1, LOT: 10141287) was acquired from Alfa Aesar, Ward Hill, MA. The OTC product was Aleve® Liquid Gels, 220mg Liquid-Filled Capsules, distributed by Bayer Healthcare LLC, Whippany, NJ (LOT: NAA4PNX, EXP: 06/18).

As with IBP, NAP is a NSAID that interacts with COX-1 and COX-2 enzymes in humans and animals, inhibiting inflammation, reducing fever and providing pain relief (Rainsford, 2015).

Acetaminophen (APAP), *N*-Acetyl-4-aminophenol, was used in two forms. Pharmacological grade material (>98.0, CAS# 103-90-2, LOT: MKBX4982V) was acquired from Sigma-Aldrich, St. Louis, MO. The OTC product was Tylenol® Liquid Gels, 325mg Liquid-Filled Capsules, distributed by McNEIL-PPC, Inc., Fort Washington, PA (LOT: 1437837, EXP: 07/18).

APAP is not classified as a NSAID. Previous studies have speculated on the mode of action as having effects on the eicosanoid, endocannabinoid, serotonergic, and nitric oxide pathways to produce the analgesic effect. Similar to IBP and NAP, APAP may interact with cyclooxygenase enzymes to produce an analogous pain relieving effect (Mazaleuskaya et al 2015).

The pharmacological grade stock solutions were prepared on a weight basis adding granulated IBP and NAP, respectively, to buffered (~pH 7.5-8) 10% SPM to achieve a stock concentration of 1mg/mL in a 50mL volumetric flask. The APAP stock solution was prepared in a similar fashion at a concentration of 2mg/mL. Prior to final dilution in a 50 mL volumetric flask, toxicant was added to approximately 30mL of 10% SPM, and contents were stirred for 30 min to assure dissolution. The pH was adjusted, if necessary, to 7.5-8.

OTC stock solutions were prepared similarly by obtaining the average weight of the liquid contents of eight individual liquid-filled capsules. For ibuprofen, 200mg IBP liquid-filled capsules were used (429mg). For naproxen, 220mg NAP liquid-filled capsules were used (849mg). For acetaminophen, 325mg APAP liquid-filled capsules were used (967mg). The stock solution was created on a weight basis by expelling and weighing the liquid contents of the capsules prior to dilution in buffered (~pH 7.5-8) 10% SPM to obtain an estimated concentration of 1mg/mL for IBP and NAP and 2mg/mL for APAP in a 50mL volumetric flask. As with the pharmaceutical grade material, OTC material was added to approximately 30mL of 10% SPM,

and contents were stirred for 30 min to assure dissolution prior to final dilution in a 50 mL volumetric flask. The pH was measured and adjusted, if necessary, to 7.5-8.

Copper (II) (as $CuSO_4 \cdot 5H_2O$) was tested to assess response to a known metal toxicant. The copper stock solution was made using a 10% SPM. To make the primary stock solution 0.1964g $CuSO_4 \cdot 5H_2O$ was added to approximately 20 ml of 10% SPM and mixed for approximately 25 min. The pH was 4.17 and was not adjusted, as solubility was not an issue. The solution was then transferred into a 50ml volumetric flask and diluted with 10% SPM to 50 ml. A secondary stock solution was made by diluting the primary stock to 1 µg Cu^{+2} mL^{-1} using 50 µl of primary stock and 10% SPM in a 50 mL volumetric flask.

Cell counts were transformed to percent of control response by dividing each replicate cell count by the control mean for each respective experiment. The dose-response relationship was examined using linear regression of transformed cell count data and log dose. Comparisons among experimental treatments were also analyzed using analysis of variance (ANOVA) followed by Dunnett's test to identify treatments differing from controls (Dunnett 1955). This analysis was used to define the no observable effect concentrations (NOEC) and lowest observable effect concentrations (LOEC) for each experiment, where "effect" was defined as an adverse response (growth inhibition) relative to controls. Regression and inverse prediction were used to determine both the EC_{20} and EC_{50} of each toxicant (Sokal and Rohlf 1981). A 95% confidence interval for the EC_{20} was estimated based on regression results. Statistical analyses were performed using Minitab Express™ Version 1.5.0 (Minitab Inc, State College, PA, USA).

RESULTS AND DISCUSSION

Experiments showed strong dose responses and similar toxicity estimates for OTC and pharmaceutical grade material. In general, the OTC materials were less toxic than the pharmaceutical grade material (Fig 1, 2; Table 1). This was expected, since the OTC material was extracted from liquid-filled capsules and had more variability than the pharmaceutical grade compound. It is also likely that the assumed toxicant content of those capsules was not achieved in making the stock solutions. All analyses were based on nominal toxicant concentrations.

Dose responses were linear with log dose and all r-square estimates were greater than 0.75 and most greater than 0.80. Estimates of confidence intervals for the EC20 endpoints often overlapped, suggesting that both the pharmaceutical grade material and OTC material produced similar toxic responses. IBU was more toxic to *C. steinii* than either NAP or APAP (Table 1). These results correspond to the hazard ranking from other measures of toxicity of these PPCP's.

Copper toxicity (Table 1, as Cu^{+2}) was similar to estimates for other experiments using colpodid ciliates and overlapped the range of reported copper toxicity in other rapid bioassays. Pratt et al (1998) showed copper toxicity to *Colpoda inflata* was approximately 0.575 mgL^{-1} as EC50 in 5% Sonneborn's medium and 0.030 mgL^{-1} in a minimal salts medium. Forge et al (1993) showed similar results (EC50 0.025 mgL^{-1}) for Cu^{+2} tests conducted with *C. steinii* in minimal salts medium. Roberts and Berk (1990) reported an EC50 of 0.045 mgL^{-1} for Cu^{+2} in a one-hour chemoattraction assay using *Tetrahymena pyriformis*. These results suggest that the sensitivity of the current *C. steinii* test is comparable to other toxicity estimates, although dissolved organic matter in the medium is well-known in binding metal toxicants and reducing their apparent toxicity (McIntyre and Gueguen 2013).

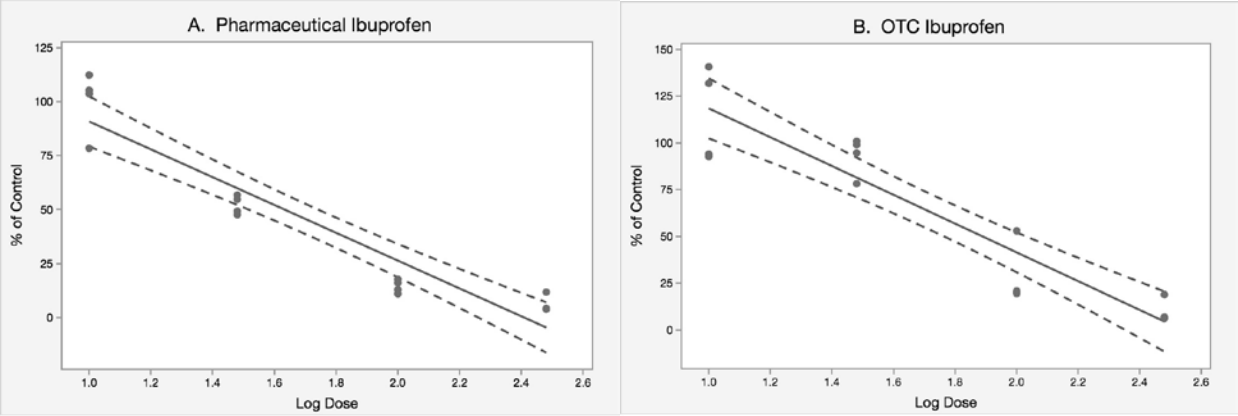

Fig 1 Effect of Pharmaceutical (A) and OTC (B) Ibuprofen on *C. steinii*. Data are expressed as percent of control. Test concentrations ranged from 10 mgL^{-1} to 300 mgL^{-1}. The fitted line is from linear regression (95% confidence interval indicated by the dashed line).

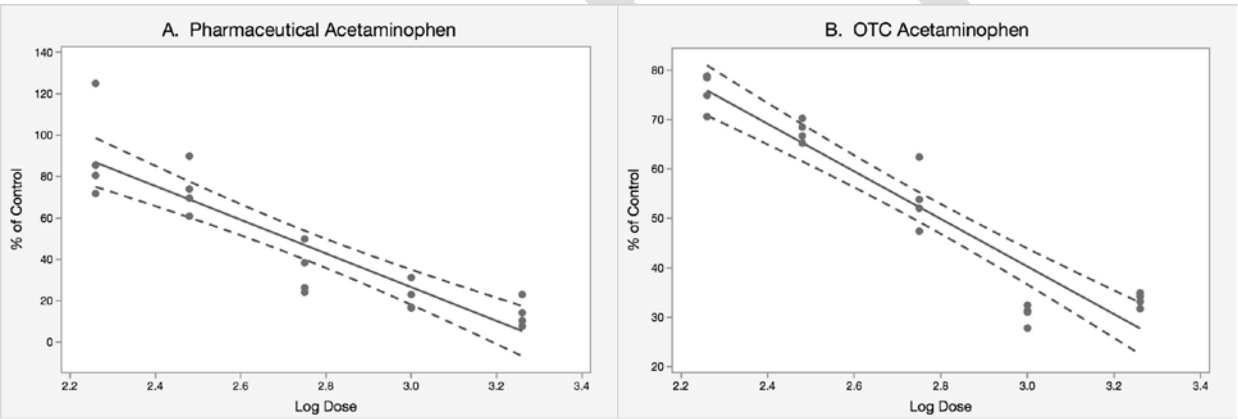

Fig 2 Effect of Pharmaceutical (A) and OTC (B) Acetaminophen on *C. steinii*. Data are expressed as percent of control based on the mean control response. Test concentrations ranged from 180 mgL^{-1} to 1800 mgL^{-1}. The fitted line is from linear regression (95% confidence interval indicated by the dashed line).

Table 1 summarizes toxicological endpoints from the experiments. The EC_{20} is the concentration at which there was 20% inhibition of growth relative to controls. Similarly, the EC_{50} is an estimate of the toxicant concentration corresponding to a 50% inhibition of growth. The NOEC and LOEC are based on multiple comparisons of treatments to control in each experiment ($p<0.05$, Dunnet's test) and identified the highest test concentration not different from controls (NOEC) and the lowest test concentration differing from controls (LOEC). While these values compare favorably to the EC20 and EC50 estimates, they are limited by the doses chosen for the experiment.

Table 1 Summary of toxicological endpoints (data are mgL^{-1}). EC_{20} and EC_{50} values were determined using inverse prediction. Corresponding r^2 values for the dose-response relationships are shown ($p<0.001$) along with the coefficient of variation (CV) for controls in each test. NOEC – no observed effects concentration; LOEC – lowest observed effects concentration.

TOXICANT	NOEC	LOEC	EC_{20}	EC_{20} 95% CI	EC_{50}	R^2	CV
IBUPROFEN							
PHARMACEUTICAL	10	30	15	(12 - 19)	43	0.896	12.39%
OTC	30	100	32	(23 - 48)	78	0.866	11.34%
NAPROXEN							
PHARMACEUTICAL	100	180	132	(96 - 195)	251	0.912	6.95%
OTC	180	300	191	(102 - 437)	398	0.75	11.47%
ACETAMINOPHEN							
PHARMACEUTICAL	300	560	219	(126 - 437)	513	0.811	17.95%
OTC	N/A	180	148	(229 - 776)	631	0.89	8.80%
COPPER (Cu^{++})	0.30	0.56	0.21	(0.11-0.48)	0.40	0.767	4.9%

In a study of 26 PPCP's, Ortiz de Garcia, et al (2014), compared six short-term toxicity endpoints to determine their order of sensitivity. Experiments included two Microtox® tests (5 min. and 15 min.), and activated sludge respirometry. Toxicity was inferred for a 96-hour green algae test, a 48-hour *Daphnia magna* test, and a 96-hour fish test based on octanol-water partition coefficients of the PCPP's. The EC_{50} of each toxicant was estimated and results showed the same range of toxicity values for ibuprofen, naproxen, and acetaminophen.

A 48-hour study of growth inhibition using neonatal *D. magna* reported EC_{20} results (mgL^{-1}) and 95% confidence interval for ibuprofen (76.4, 62.9–92.9) and naproxen (64.8, 39.9–105.3), similar to *C. steinii* results reported here (Cleuvers 2004). This test utilized an artificial medium (ADaM, Aachener Daphnien Medium) that mimics natural freshwater (Klüttgen et al 1994). Separate 48-hour survival studies of *D. magna* using acetaminophen showed EC_{50}'s of 30.1 mgL^{-1} (23.2–39.0) (Kim et al 2007) and 11.85 mgL^{-1} (8.57–16.38) (Kim et al 2012). Each of these studies reported much lower EC_{50} values than the *C. steinii* microscale test. Both studies utilized purified water as the test medium.

As it relates to the chemicals studied, *C. steinii* bioassay is sufficiently sensitive to these toxicants when compared to other studies. Thousands of PPCP chemicals have yet to be examined for their effects on aquatic biota and ecosystems. These tests could prove a useful tool in identifying new and varied toxicological endpoints (Wells et al 1998). Inexpensive, reliable, and rapid bioassays, as the one used in this experiment, could help to prioritize PPCP's that are found in higher quantities in the aquatic environment or those that have high toxicity.

Cyst-based ciliate bioassay use in acute screening has been studied against traditional priority pollutants, and their role is clear. With some exceptions (e.g. some pesticides), ciliate tests can be as sensitive as standard *Daphnia* and fish bioassays (Wells et al 1998). What is unknown are the effects of emerging PPCP's on these organisms. In an attempt to streamline these tests, differing variables have been explored. Experiments can be done using varying media, such as minimal salts media to the defined media used in this study. Test materials may

include 24-well polystyrene plates (Pratt et al 1997) or culture tubes (Pauli and Berger 1997). With the short generation time of these organisms, most studies run from 24-96 hours. With further testing and adjustment, microscale bioassays that utilize cyst forming organisms can be an important tool.

The variability of the growth response can be estimated from the coefficient of variation (CV) of controls for each test. In this examination log-phase growth was weak at 24 hours but robust enough at 48 hours to yield approximately 200 cells per 10µL given a standard inoculum of approximately 100 cells/2mL. Understanding the CV of the controls would assist investigators in determining the power of the experiment. Table 1 shows the CV for each of the tests done in this experiment and gives some information on the strength of the examinations. Considering the thousands of PPCP compounds to be studied, reporting the CV and r^2 values for the dose response will lend insight to the strength of the tests, especially when compared to studies of traditional priority pollutants.

These experiments can be strengthened in several ways, including confirming toxicant concentrations in stock solutions and examining the effects of differing test media. The 10% SPM used in this study provided adequate growth, but other media may be better suited to simulating natural waters. Using medium that more closely mimics the natural environment could advance methodology. A minimal salts medium has been used because of the binding of toxic metals to organic molecules preventing uptake (Wells et al 1998). One study examined growth kinetic characteristics of ciliates in varying media (Pauli and Berger 1997). Calibrating tests with well-studied reference toxicants is needed to further understand the range of sensitivities of the test organisms.

This study used *C. steinii* to examine the effects of three PPCP's commonly identified in water systems. The experiments offer information to compare with other acute studies to obtain a clearer picture of the potential effects of PPCP's in the environment. This test is efficient, cost-effective, and reliable. Screening tests and hazard ranking, using rapid tests such as this are important because of the complexity and multitude of PPCP's in aquatic ecosystems.

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