Measuring Biotic Ligand Model (BLM) Parameters *In Vitro*: Copper and Silver Binding to Rainbow Trout Gill Cells as Cultured Epithelia or in Suspension

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Abstract

Biotic ligand models (BLMs) for metals are useful for risk assessment. The modelling of metal complexation by the biotic ligand has received little attention relative to the modelling of organic and inorganic complexation of metals in solution. We used ion selective electrodes (ISEs) to directly characterize copper and silver binding to rainbow trout gill cells, either as cultured reconstructed epithelia , or dispersed in suspension. Preparations were composed of pavement cells (PVCs) alone or mixtures of PVCs (≈85%) and mitochondria-rich cells (MRCs, ≈15%). Mixed cells showed up to an order of magnitude greater binding for both metals, indicating that MRCs were much more important than PVCs. Also, cell orientation had a dramatic effect; cells cultured as epithelia exhibited much greater binding than cells in suspension. Silver and copper demonstrated generally similar binding behaviour, with stronger (logK≈10 or greater) and weaker binding sites (logK≈8). Comparisons to existing BLM calibrations show good agreement, but reveal that selection of analytical window can impact which binding sites are titrated. We conclude that cultured gill epithelia *in vitro* provide a powerful approach to studying metal complexation directly at the biotic ligand.

Introduction

Biotic ligand models (BLMs) are useful tools for metals risk assessment and to help establish appropriately protective ambient water criteria.[1–5] The basis of biotic ligand modelling is that metal accumulation at the "biotic ligand" is proportional to toxicity and that this accumulation can be predicted by solving the appropriate simultaneous equilibria among the biotic ligand(s) and the dissolved components (aqueous ligands and competing cations) in the exposure water. For fish toxicity prediction, the biotic ligand is most often thought of as the gill.[6] The most frequently studied aspect of the BLM equilibrium problem is the aquatic geochemical speciation of the metal of interest, i.e., calculation of aqueous organic and inorganic complexes of the metal using modelling frameworks, such as those recently

summarized by Leal et al. 2014.[7] The portion of the equilibrium model predicting metal binding to the biotic ligand has received comparatively less attention, yet is arguably the most important. Here we apply a standard experimental geochemical technique, i.e., ion selective electrode (ISE) titrations, to investigate rainbow trout gill model systems to assess equilibrium binding of gill cells in the context of biotic ligand modelling.

Ion selective electrodes are often used in titrimetry to determine binding affinities ($\log K$) and capacities for geo-reactive surfaces such as bacteria,[8] minerals[9] or natural organic matter (NOM).[10] Such electrodes have the advantage of responding to free ion concentrations (activities) and the free ion is often thought to be proportional to bioavailability.[11] The silver electrode is the most sensitive ISE[12] and has been used to determine silver binding to strong ligands in NOM.[13] The cupric ISE has been used extensively for both freshwater[14] and marine applications[15] and with sufficient buffer capacity (i.e. binding ligands) cupric electrodes can respond to very low free ion concentrations,[16] potentially as low as 10^{-19} M. To probe high affinity binding sites it is necessary to measure free ion concentrations with values within an order of magnitude of $1/K$;[17,18] thus, for the gill cell titrations performed here cupric and silver ISEs were used.

Traditionally, metal binding to fish gills has been studied by exposing intact living fish to various metal solutions for a specified length of time (often 3 or 24 hours), then harvesting the gills for metal analysis.[6,19–23] I such studies the "free" metal ion concentration is not usually directly measured; instead, it is estimated by geochemical speciation calculations that can only be as good as the input chemistry and underlying thermodynamic database. In such studies intact living fish studies, it is difficult to span a wide range of concentrations and to control the exposure water chemistry. Furthermore, organism heterogeneity often leads to a wide spread in the data, such as was observed by Janes and Playle (1995) for silver binding to rainbow trout gills. However, there are now several *in vitro* models available for rainbow trout gills in culture, including reconstructed flat epithelia cultured on filter membrane supports.[24–26] In the latter, the composition of both the apical (water-side) and

basolateral (blood-side) media can be controlled. To date, only one study has used this type of preparation, and this was to study the binding of radiolabelled silver to trout gill cells *in vitro*.[27] In the present study, we explore the use of ISE titration of four different types of trout gill cell preparations. An ISE titration of such a system facilitates a much wider range of metal additions, and thereby allows probing of the strongest to the weakest binding sites for a single population of cells.

Copper and silver are particularly interesting to compare and contrast in terms of their binding to fish gill cells. Copper and silver are both potentially toxic elements but copper is an essential nutrient[28] while silver is nonessential.[29] The toxicity of both copper and silver is thought to be ionoregulatory in nature where both ions are thought to interfere with sodium transport, as demonstrated in rainbow trout by Grosell and Wood (2002)[30] for copper, Morgan et al. (1997)[31] for silver, and Goss et al. (2011)[32] for both metals. The complexation chemistry of silver and copper is also similar with both ions having strong affinities for reduced sulfur (i.e., thiol) and amino binding sites, although silver(I), as the more soft metal, has even stronger affinity for reduced sulfur compared to the less soft cupric ion.[33] The chemical similarities are potentially even greater if copper(II) is reduced at gill surfaces to the more soft copper(I) ion, as has been suggested in the review by Grosell.[28]

There are two major cell types in fish gills, pavement cells (PVCs) and mitochondria-rich cells (MRCs). The MRCs comprise about 15% of gill cells and PVCs make up the remainder.[25] Both types of cells seem to be involved in acid-base balance and ion regulation including sodium (Na^+) chloride (Cl^-) and calcium Ca^{2+} uptake.[34] The general consensus is that the MRCs play the more important roles in these processes.[34–36] Indeed, as noted earlier, both silver and copper are very potent inhibitors of Na^+ transport in fish. However there is little information available on differences in metal binding abilities between the two types of cells. If MRCs really are more important in ionoregulation, then we would hypothesize that they would also be more important in the binding of metals such as silver and copper, which are ionoregulatory toxicants.

In this current work we present ISE-measured silver and copper binding isotherms for rainbow trout gill cells in culture. Particular goals were to measure $\log K$ and binding capacities over a wide range of metal ion concentrations, and to elucidate the relative roles of the MRCs versus the PVCs in this binding.

Experimental (Materials and Methods)

Gill Cell Preparation for Titration

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada). The fish (85-200 g) were held in dechlorinated Hamilton tap water (typical Hamilton tap water chemistry of $[Na^+]$=0.55 mM; $[Cl^-]$=0.70 mM; $[Ca^{2+}$=1.00 mM; $[Mg^{2+}]$=0.15 mM; $[K^+]$=0.05 mM; pH 7.8-8.0; dissolved organic carbon (DOC)=3.0 mg/L) at seasonal temperatures (12-15 °C).

Trout gill cells were prepared in four different ways (see below). Methods for all four procedures are detailed in Kelly et al. (2000)[37] and Schnell et al. (2016),[26] so only brief detail is provided here. In all cases, the procedures started with mixed cells obtained from the gills of a euthanised trout after digestion by treatment with a trypsinizing solution (0.05% trypsin, 0.02% EDTA in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}) and centrifugation. The pellet of cells was then washed several times with PBS containing 5% foetal bovine albumen (FBS), and finally resuspended in culture medium (Leibowitz L-15 supplemented with 2 mmol L^{-1} glutamine and 5 % FBS).

(i) *PVCs Alone in Suspension.* Procedures followed those first described by Pärt et al. (1993).[38] The dispersed cells were grown in flask culture (25 cm^2 Falcon^{TM} Cell Culture Flasks, Corning, Durham NC, USA), until they reached confluence (typically 5-7 days). During this period, the MRCs fail to attach and die out, leaving only the PVCs. The PVCs were then removed from the flasks by trypsinization as above, and resuspended in titration medium at a density of approximately 0.5 million cells per mL.

(ii) *Mixed Cells in Suspension.* The original freshly prepared mixed cells were resuspended in titration medium at a density of $\sim$ 0.5 million cells per mL.

(iii) *PVCs Alone as Reconstructed Epithelia.* Procedures followed those first described by Wood and Pärt (1997)[39] so as to create single-seeded insert (SSI) preparations. PVCs were first grown in flask culture until they reached confluence, then removed by trypsinization as described in (i) above. The resuspended cells (pure PVCs) were seeded onto the upper surface of permeable Falcon^{TM} filters [0.45 mm pore size, low pore density (1.6×10^6 pores cm^{-2}), 4.30 cm^2 area Cyclopore Polyethylene Terephthalate Filter Inserts, Becton Dickinson, Franklin Lakes, New Jersey, USA]. Seeding density was 0.5 $\times$ 10^6 cm^{-2}. The inserts were seated in Falcon^{TM} cell culture companion plates, and both the upper (apical) and lower (basolateral) media were L-15 supplemented with 2 mmol L^{-1} glutamine and 5 % FBS. Transepithelial resistance (TER) was monitored daily using STX-2 chopstick electrodes connected to a custom-modified EVOM epithelial volt/ohmeter (World Precision Instruments, Sarasota, Florida, USA). Once a stable value was reached ($>$ 10,000 ohms cm^2), generally after 6-9 days, the apical surface of the epithelium was washed three times with titration medium, while L-15 + 2 mmol L^{-1} glutamine + 5 % FBS remained as the basolateral medium. The preparation was then used in experiments.

(iv) *Mixed Cells as Reconstructed Epithelia.* Procedures followed those first described by Fletcher et al. (2000)[40] so as to create double-seeded insert (DSI) preparations. The first seeding of original freshly prepared mixed cells onto the filter inserts was done at very high density (2.0–2.5 $\times$ 10^6 cells cm^{-2}), soon after they were isolated (day 1). On day 2, the apical surface of the preparation was washed 3 times with the culture medium, and then a second seeding of freshly prepared mixed cells from a different trout was performed, again at very high density. In this protocol, most of the cells from the first seeding fail to attach and are subsequently washed away, but a small

percentage of the PVCs do attach and persist. These create a “lawn” which offers an appropriate environment for incorporation of MRCs from the second seeding. L-15 + 2 mmol L^{-1} glutamine + 5 % FBS remained on the basolateral surface. Thereafter, TER was monitored daily as in (iii) until a stable value was reached ($>$ 15,000 ohms cm^2), generally after 6-9 days. The apical surface of the epithelium was then washed three times with titration medium, as in (iii), after which the preparation was ready for use in experiments.

In all four protocols, once the titrations were finished, the cells were trypsinated (if necessary), and an estimate of the total number of cells in the preparation was obtained by counting a subsample in a haemocytometer grid.

Titration Methods

All chemicals used in titrimetry were 99.9% pure or greater. Acid-washed plastic and glass ware was used throughout. Acid-washing involved at least 24 hours soaking in 10% trace metal grade nitric acid followed by extensive rinsing with ultrapure water ($\leq$ 18.2 MΩ resistance, MilliQ water, EMD MilliPore, Etobicoke, ON, Canada). All titrations were performed in at least duplicate and data from replicate titrations are pooled for parameter fitting (see below). Titrations were performed at room temperature (21 °C).

Prior to titration, the Cu (Orion Model 94-29, Boston, MA, USA) and Ag (Metrohm, Model 6.0502.180, Sweden) ISEs were polished to a "mirror-like" finish using aluminum oxide ($<$ 10 micron, 99.7%, Sigma Aldrich, St. Louis, MO, USA) followed by silver electrode polish (Corning Inc, Tewksbury, MA, USA). An Orion double junction Ag/AgCl reference electrode (Model 900200, Boston, MA, USA) was used as reference and potential recorded on a Tanager potentiometer (Model 9501, Ancaster, ON, Canada). To ensure stable readings a “Faraday cage” was created by wrapping the titration cell with a grounded wire, attached to the building water pipe. During measurement of cell suspensions the solutions were stirred mechanically with a magnetic stirrer, for epithelia the solutions were mixed on the apical

side using a recirculating peristaltic pump.

The same basic method was used for all titrations. After addition of titrant, the electrode potential was monitored until it was stable. The stability criterion was defined as a random drift of less than 0.1 mV/min for at least a 5-min observation period. Generally signal stability was reached within 15-30 min after titrant addition, but sometimes equilibration times as long as 2 h were necessary for a single titration data point. The entire titration curve was obtained within approximately 10 h of initiation of the experiment. During the entire titration, pH was monitored using a glass electrode (Combination pH Electrode, Epoxy Body model no.815600/34107-377, Thermo Fisher Scientific, Mississauga, ON, Canada) and manually adjusted using dilute acid and base (HNO_3 and NaOH respectively, Sigma-Aldrich, Oakville, ON, Canada) to maintain pH at 7.70 ± 0.03. After the final titrant addition, the pH of the sample was adjusted to approximately 4.5 and the stable mV value recorded using the same stability criteria noted above. This single measured potential was used as an internal standard to calibrate the electrode for that specific titration (see below).

Silver (as $AgNO_3$ from Sigma-Aldrich, Oakville, ON, Canada) and copper (as $CuSO_4$ from BioShop Canada Inc., Burlington, ON, Canada) titrants were prepared at 100 and 1000 μg/L. The specific stock solution used for any given addition depended on the specific target total metal concentration. The concentration of total metal in solution was increased in steps during the titration to achieve approximately equal $\log[M^{n+}]$ intervals in the measured free ion. Fresh titrant solutions were prepared each day to avoid sorptive losses to the storage vessel walls. To avoid wide changes in pH of the titration solution the pH of the stock metal titrant solutions were adjusted to approximately 4.0 using dilute nitric acid.

Electrode potential is linearly related to the base ten logarithm of concentration of free metal ion according to the Nernst equation.[12] A one point internal calibration procedure was used to determine the intercept of the Nernst equation. For comparison, calibration slopes and intercepts were also determined in standard solutions prior to each titration. Standard solutions for copper were prepared using etheylene diamine, as in Tait et al.,[15] and

for silver, halide (KI, KBr, KCl) standard solutions were used as in Smith et al. (2004).[13] Externally determined slopes and intercepts were within 10% of the internally determined values using one-point calibration. For the one point calibration method, a Nernstian slope of 29.6 for Cu^{2+} and 59.2 mV per decade for Ag^{+} was assumed (theoretical Nernstian slopes for a divalent and monvalent cation respectively, at 25 °C). This internal standard one-point calibration approach was developed in a recent paper by Tait et al. (2015) and demonstrated improved reproducibility compared to traditional external calibration methods.[15] By using the response in the actual titration solution to calibrate the electrode, any matrix effects and sample-specific bias is corrected; in addition, since the ionic strength is fixed (see below), the calibration is done in terms of concentration as opposed to activity units. At the final acidic measured potential it is assumed that total copper or silver are equal to free copper or silver ion plus any inorganic complexes [e.g., the chloride complexes as determined using NIST (National Institute of Standards and Technology) $\log K$ values,[41] see Supplementary Information]. Organic binding should be negligible at low pH, as demonstrated for Suwannee River Fulvic acid and copper by Cabaniss and Shuman.[42] Note, for the calibration potential reading, the pH must not be below 4.0 because because ISEs start to respond to protons at low pH.[14]

Total volumes were 25 mL for suspension solutions and 7 mL for titrations of the apical surfaces of inserts. The volume change over the course of a titration was less then 1 and 5% for suspension and insert titrations respectively. For copper titrations, both for suspension and the apical solution of insert titrations, the solution composition was 150 mM NaCl and 2 mM $CaCl_2$ (salts from Sigma-Aldrich, Oakville, ON, Canada). Thus the ionic strength was fixed at approximately 0.156 mol/L. For silver titrations the chloride salts were replaced with gluconate salts (Sigma-Aldrich, Oakville, ON, Canada). It was necessary to use gluconate instead of chloride as the anion to avoid silver chloride precipitation. For gill epithelia titrations there were approximately 1×10^6 cells per insert and suspensions included approximately 1×10^7 cells in each titration sample.

Blank titrations of copper showed negligible binding to glassware or inserts. For silver some binding was observed during blank insert titrations; such binding was also observed in the Zhou et al. study.[27] To correct for this binding, blank titration were fitted to a one site Langmuir isotherm as a function of the free ion. The corresponding equation (support-bound silver = (K×[Ag^+]×capacity)/(1+K×[Ag^+]), where logK=7.7 and capacity=8.0×10^{-5} mol/L) allowed for correction of silver not bound to gill cells during sample titrations. In this way the measured free ion was used to predict the "support-bound" silver, and this value was subtracted from the measured bound silver in actual cultured gill titrations. All results presented below have had this correction applied but similar to the observations of Zhou et al.[27] in the presence of gill cells on the membrane supports, the binding to the filter was less than 1%.

Titration Data Modelling Methods

All data analysis was performed in Matlab ™ (MathWorks Inc., MA, USA). For modelling of titration data the initial known total metal ($[M_T]$ and measured free ion ($[M^{n+}]$, where n=1 for Ag and 2 for Cu ions) were used to calculate cell-bound metal ($[M_{bound}]$) according to Equation 1.

$$[M_{bound}] = M_T - [M^{n+}] - \sum_{i=1}^{m} \text{inorganic bound metal} \quad (1)$$

Note: bound metal refers to all metal removed from solution and could include surface complexes as well as metal taken up into the cell. The sum of inorganic bound metal for m different inorganic anions was calculated using the NIST certified $\log K$ values (see Supplementary Information for details). For the purposes of modelling, the data were normalized to the number of cells, so the fitted binding site densities were also determined per cell. The final data in the form of free metal ion ($[Cu^{2+}]$ or $[Ag^+]$) *versus* bound metal (in mol/cell), were fitted to a multi-site Langmuir equation. Equation 2 corresponds to a three

site Langmuir equation.

$$[\mathrm{M_{bound}}] = \frac{K_1 L_{T,1}[\mathrm{M^{n+}}]}{1 + K_1[\mathrm{M^{n+}}]} + \frac{K_2 L_{T,2}[\mathrm{M^{n+}}]}{1 + K_2[\mathrm{M^{n+}}]} + \frac{K_3 L_{T,3}[\mathrm{M^{n+}}]}{1 + K_3[\mathrm{M^{n+}}]} \quad (2)$$

Thus, fitting the data involved determination of binding strengths ($\log K_p$) and capacities ($L_{T,p}$) values where p (the number of binding sites) ranged from 1 up to 3. The number of binding sites was determined as the minimum number of sites required to fit the data without trends in the residuals (i.e., the difference between predicted and observed bound metal) based on visual inspection of a plot of residuals versus $\log [\mathrm{M^{n+}}]$. The fit $\log K$ values are conditional on ionic strength and pH of the exposure media. When data spanned many orders of magnitude in free ion, multiple binding sites were necessary to account for the measured binding curves. Confidence intervals were determined about the models and best-fit parameters using Monte Carlo analysis.[17] One thousand random data sets were generated from the initial best fit model replacing a random ⅓ of the data with simulated data based on adding random error to the calculated best-fit values. Each data set was fitted and the spread of the fits and the spread about the parameter estimates were used to calculate the 95% confidence intervals about the model and the parameters respectively.

Results and Discussion

Comparison of Cu and Ag Titrations, and of Titrations of Different Cell Preparations

For titration of suspensions of PVCs, silver and copper behaved very similarly with extensive overlap between the titration curves (Figure 1 green and blue circles). The silver data extended to lower free ion concentrations (as low as 10^{-14} mol/L) and the copper titrations to higher values ($> 10^{-6}$) but in the intermediate range where they overlap the two titrations show essentially the same amount (mol) of metal bound per cell. This suggests that the

functional groups binding silver and copper in pavement cells are the same, at least in the range of free metal from 10^{-10} to 10^{-7} M. Silver titrations extended to lower free ion concentrations because the silver ISE is more sensitive than the cupric ISE.[12] Although Figure 2 shows that this is not always the case and some cupric measurements here were more sensitive than the corresponding silver measurements.

Similarly, for mixed cell preparations (i.e., $\sim$ 85% PVCs and $\sim$ 15% MRCs), the copper and silver data overlapped at intermediate free ion concentrations (10^{-9} to almost 10^{-6} M) with silver again showing a lower detection limit and wider detection range (Figure 1, red and green circles). The binding of both copper and silver in mixed cell titrations were consistently higher than in PVC alone titrations with almost an order of magnitude greater binding per cell at the lower range of measured free silver ion. For copper, and the higher end of the silver titration, the mixed cells still showed consistently higher binding than PVCs alone but by a lower factor (less than half an order of magnitude difference). Overall, these data suggest much higher binding capacities for both silver and copper in MRCs than in the PVCs.

Titration data for simulated epithelia showed less quantitative similarity between copper and silver (Figure 2). Silver (red and green data) demonstrated dramatically higher binding amounts compared to copper, with separations as great as two orders of magnitude between PVC alone preparations. Similar to the suspension data though, the mixed cell reconstructed epithelia, which included MRCs, demonstrated consistently higher binding (Figure 2); for example, PVC alone titrations demonstrated bindings which were 0.7 of an order of magnitude lower than those in mixed cell preparations in the 10^{-8} to 10^{-6} M range of the titration curve. These results are consistent with the postulated role of MRCs in metal ion transport.

Cells cultured as simulated epithelia on membrane inserts showed considerably more metal binding per cell than randomly dispersed cells in suspension; the data ranged from 10^{-16} to 10^{-13} for suspension titrations (Figure 1) but ranged up to $> 10^{-12}$ mol/cell for insert titrations (Figure 2). Thus, the binding of metals is dependent on cell orientation with

increased binding being observed when cells have specific apical and basolateral sides. This increased binding seems to occur even though nominally some binding sites are "removed" as the basolateral side (bottom of the cells) are not exposed directly to the exposure (apical) solution containing metal. For higher values of free copper though the suspension and epithelial cells had very similar binding (Figure 3).

As outlined in the Introduction, this conclusion as to the greater importance of MRCs fits with the general belief that ionoregulatory toxicants, such as silver and copper, which preferentially target Na^+ transport, will be preferentially taken up by MRCs. Indeed silver-staining is a technique which has classically been used to identify MRCs.[43,44] However the present investigation appears to be the first to actually demonstrate this difference for both copper and silver in a quantitative fashion. Nevertheless, we must note that the only previous study of which we are aware that attempted to quantify the role of the MRCs in copper uptake in fact concluded that the MRCs did not play an important role.[45] However, the conclusion of that study was also based on indirect evidence, specifically that MRC proliferation did not result in elevated Cu uptake by the intact freshwater trout gills.

Parameter Fitting Results

Modelling was performed to facilitate quantitative comparisons among data sets. These modelling parameters are also useful in that they can suggest potentially the types of functional groups involved in metal binding over specific ranges of free ion concentration. Figure 4 presents model best-fit lines, and associated 95% confidence intervals for silver titrations. Some of the measured data were too steep to fit with a Langmuir binding-site model. These data were excluded from data fitting (see Figure 4). Likely these data points were below detection. For some unknown reason the cells prepared as simulated ephithelia did not buffer the silver ion as well as suspensions of cells. An ISE measured response (potential) is proportional to log of the ion concentration; thus, it is impossible to measure "zero" concentration, and instead a constant free ion concentration is observed at levels at or below the detection

limit.

For silver, it is clear that the reconstructed gill epithelia showed greater metal binding per cell than the corresponding cell suspensions. Oriented cells, in the epithelia, bind more silver than distributed cells in suspension, even though the reconstructed epithelia are typically 2-5 cell layers thick as *in vivo*, such that only 20-50% of the cells may have direct exposure to the apical solution.[26,39,40] In contrast in suspension preparations, the entire surface of all cells would be exposed, including basolateral cell membranes which would normally not be exposed to the external medium *in vivo*. In the reconstructed epithelia, as *in vivo*, cells are designed to have a "top" and a "bottom" and ion transport processes take advantage of the gradient, and the ordered transport pathways (e.g. apical channels, basolateral transport enzymes), between the apical and basolateral sides of the epithelium. This seems to be the case for metal ion binding as well.

For copper, this tendency for higher binding by oriented cells in reconstructed epithelia *versus* cell suspensions was also observed, to a lesser extent then for silver, in mixed cell preparations (PVCs and MRCs, Figure 3). In gill epithelia titrations copper actually exhibited a lower detection limit than in suspensions, presumably because of the greater buffer intensity provided by a larger abundance of ligands. Interestingly, PVC alone titrations showed much less difference between suspensions and reconstructed gill epithelia, with the epithelia titrations actually showing less binding per cell than was observed in suspension, at least at lower free ion concentrations (Figure 3). At the higher end of the titration curve (10^{-8} to 10^{-6} M range), the suspended PVCs showed the same copper binding per cell as PVCs incorporated into an epithelium.

Figures 4 and 3 have inset plots showing calculated MRC alone binding isotherms compared to modelled PVC alone isotherms. The MRC alone isotherms were determined by assuming the model-fit mixture corresponded to 85% PVCs and 15% MRCs. Thus, the MRC isotherm is determined as the mixture isotherm minus 0.85 times the PVC isotherm, all divided by 0.15. These inset plots demonstrate the same tendency of greater binding in

MRCs than in PVCs.

Fitting parameters are summarized in Table 1. For silver, the range of $\log K$ values fall into classes of stronger (site 1, $\log K > \approx 10$), medium (site 2, with $\log K$ value between 8.2 and 10) and weaker (site 3, $\log K < 8.2$) binding. Although copper, with a more limited titration range due to the detection limit of the ISE, showed at most only two binding sites there is still a clear tendency for stronger (site 1, $\log K > 10$) and weaker (site 2, $\log K \approx 8$) binding. These observations of stronger lower concentration sites and weaker higher concentration binding sites, are consistent with Town and Filella testing of an "L1 L2" model for metal binding to natural organic matter.[46]

The observation from the suspension cell titration data for PVCs alone, that copper and silver seem to share common binding sites, is supported by the parameter fitting results. The weaker copper binding site ($\log K$=8.0) has a $\log K$ 95% confidence interval that overlaps with the weaker binding site for silver ($\log K$=9.1). Not only are K values similar but concentrations of binding sites suggest both metals could interact at a common site; the lower confidence interval was observed as 37.7 fmol/cell for copper and an upper end of 35.5 fmol/cell for silver. The stronger binding sites had very similar $\log K$ values as well (11.1 for Cu and 12.4 for Ag). It is very reasonable that sites that bind silver strongly will also bind copper strongly, as mentioned in the Introduction, especially if Cu(II) is reduced to Cu(I). With $\log K$ values in the 11-12 range it is likely that the metal binding site is a thiol, demonstrating cysteine-like binding.[33] For mixed suspensions, only the weaker site was observed in the detection limit range for copper ($\log K = 8.1$) but for silver strong, medium and weaker binding sites were determined ($\log K$ values of 12.9, 9.9 and 6.1). The copper binding capacity determined for this one site (around 100 fmol/cell) was greater than the 34 fmol/cell determined by combining both the stronger and medium binding site densities for silver.

For titrations of reconstructed epithelia, the same general trend is observed with a range from stronger to weaker metal binding sites; for example, for mixed cell epithelia, the

strong/weak $logK$ values are 10.8/8.5 for copper and 9.8/8.2 for silver, and the weaker site 2, 95% confidence intervals, again overlap as was observed for suspended cells. Likely the binding sites in the 8 range are related to amine sites.[33] However, the binding capacities are dramatically greater for silver than for copper (1000s of fmol/cell compared to 10-100s). This is true for both epithelia comprised of PVCs only and for mixed cell epithelia. In contrast, the difference in binding capacities for silver *versus* copper was not readily apparent in the titration data from cell suspensions. These comparisons again highlight the difference between cell metal binding for oriented cells in epithelia compared to dispersed cells in suspension. In addition to differences due to lack of orientation and "inappropriate" exposure of the basolateral surfaces mentioned earlier, the cells in suspension may well have lost a significant number of their normal surface proteins because they had only a few hours to recover from trypsination, in contrast to the 6-9 day recovery period for reconstructed epithelia.

To facilitate comparisons between PVC and MRC metal binding, the calculated MRC titration curves, see insets graphs on Figures 4 and 3, were fitted to Langmuir isotherms in the same way as the original experimental data. Note, no binding site 2 was determined for silver because the negative data resulting from subtraction of measured PVC data from measured MRC data was omitted (red dashed lines on inset in Figure 4). It is clear from comparing this MRC binding parameter data to PVC data that MRCs have dramatically higher binding capacities than PVCs by an order of magnitude or more. The K values are very similar between PVCs and MRCs though and it seems the major difference between cell types is the abundance of metal binding sites. That similar sites occur on both cells is not surprising because PVCs and MRCs have both been proposed to be involved in ion regulation[34] although it is generally accepted that MRCs play a more important role in this process.[34–36]

Comparison to Existing BLMs

The $\log K$ values determined here are conditional on pH and ionic strength (Ca^{2+} and Na^{+} competition in particular). The thermodynamic value would correct for these effects but would require experimentation varying the pH and ionic strength outside the physiological range that the cells can tolerate. The conditional values can be compared to literature values though as many of the literature studies used conditions not completely dissimilar to the isotonic solutions used for this current study.

The potential for a specific free ion concentration to exist is directly related to the $\log K$ value of the metal biotic ligand complexation reaction. By rearranging the one to one metal ligand association constant, it is possible to see that the metal-bound and free forms of a biotic ligand site are equal when the free metal ion is equal to $1/K$ (or $\log[M^{n+}] = -\log K$). Since free ion is thought to be significant in controlling toxicity[11] the most relevant BLM parameter to compare these current best fit parameters to is the $logK$ value (Table 1). For copper, BLMs for trout available in the literature have $\log K$ values of 7.4[2] and 7.6,[23] which are very similar to the Cu binding site 2 values measured here in the range 7.7-8.9. It seems likely that the most relevant site influencing copper toxicity to rainbow trout has a $\log K$ value around 8.0, and that this is the site that has been characterized in the past studies. Indeed, a study on isolated but intact trout gills exposed *in vivo* yielded a $\log K$ value of 8.1 for copper binding.[47] It does seem that there is also at least one stronger binding site with $\log K$ in the 10 to 11 range (Table 1). This site occurs at lower concentrations than the weaker BLM site with total binding densities in the range 4 to 66 fmol/cell range across all the titrations, compared to 17 to 140 fmol/cell for the weaker binding site. Notably, Taylor et al. (2000)[48] reported a $\log K$ for copper of 9.2 for intact trout acclimated to hard water, which may have reflected the influence of higher affinity binding sites due to genetic variation between rainbow strains or possibly because they were performing measurements on gills in living trout as opposed to cell cultures.

For silver BLM literature, reviewed by McGeer et al. (2000),[49] $\log K$ values range from

10 to 7.3 (Table 1). The higher $\log K$ value was obtained from gill binding experiments performed with whole fish[6] and the lower $\log K$ values (mid 7 range) were determined from parameter fitting of toxicity studies. Interestingly, an intermediate $\log K$ value of 8.8 was obtained by Zhou et al. (2005)[27] using the same reconstructed mixed cell epithelium as a model gill, prepared exactly as in this current work (DSI preparation). In that study, the $\log K$ value was calculated from the concentration-dependent kinetics of radiolabelled ^{110m}Ag binding. It seems that both previous studies (Janes and Playle[6] and Zhou et al.[27]) yield reasonable values compared to this current work where, the Janes and Playle[6] study probed the stronger site, labelled site 1 in this current study (best fit $\log K$ in the range 9.8-12.4). The weaker site probed by Zhou et al.[27] is consistent with site 2 determined here in the $\log K$ range 8.2 to 9.9 for epithelial preparations. Equilibrium constant estimates can only be made if free ion, as $\log[M^{n+}]$, is in the range (within an order of magnitude) of the $-\log K$ value. By selection of a range in total metal concentrations in those studies, the two previous studies each probed only one of the binding sites observed in this current work. The lower $\log K$ values (mid 7 range) derived from fitting toxicity data probably reflect the mixed physiological processes leading to mortality, as fish exposed to the same concentration do not all die at the same time, this would result in a wide range of concentrations on the gills.

The binding capacities determined here appear to be much higher than previous literature estimates though. Gill copper binding capacities for BLMs have been estimated in the 0.8-1.2 fmol/cell range which is much less than the 10s of fmol/cell capacity observed for Cu here (Table 1). In Table 1 the literature binding capacities, originally reported in mol per gram of gill, were converted to mol per cell by assuming 25 million cells per gram of gill, as typically observed in our laboratory after trypsination of rainbow trout gills. For silver the difference is even more extreme, with capacity estimates from the literature between 0.001 and 0.5 fmol/cell, but here estimates up to 1000s of fmol/cell (Table 1). Some of these differences might be attributed to this current work examining only the epithelial cells, and

not the whole gill, and including all metal binding where the *in vivo* experimental protocols involve the whole gill, and usually include various rinsing steps before analysis. For example, Tao et al. (2006) in studying the speciation of gill bound copper demonstrated that 45% of copper was washed from carp gills during an aqueous rinse step[50] and other washes with magnesium chloride and acetic acid removed all but 21%. In terms of toxicity prediction, and actually running BLM software code, the absolute value of the binding capacity is much less important than the $\log K$ value. Toxicity is often determined from an LA_{50} parameter (lethal accumulation to kill half the organisms) which is scaled to match species sensitivity. The binding capacity is included in the mass balance for the BLM chemical equilibrium calculation but in scheme of the total moles of metal only a very small fraction, compared to total metal concentration, actually binds to the biotic ligand surface.

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Supporting Information Available

The following files are available free of charge.

Supplementary information include a description of how total metal was corrected for inorganic complexation during calibration and cell binding isotherm determination.

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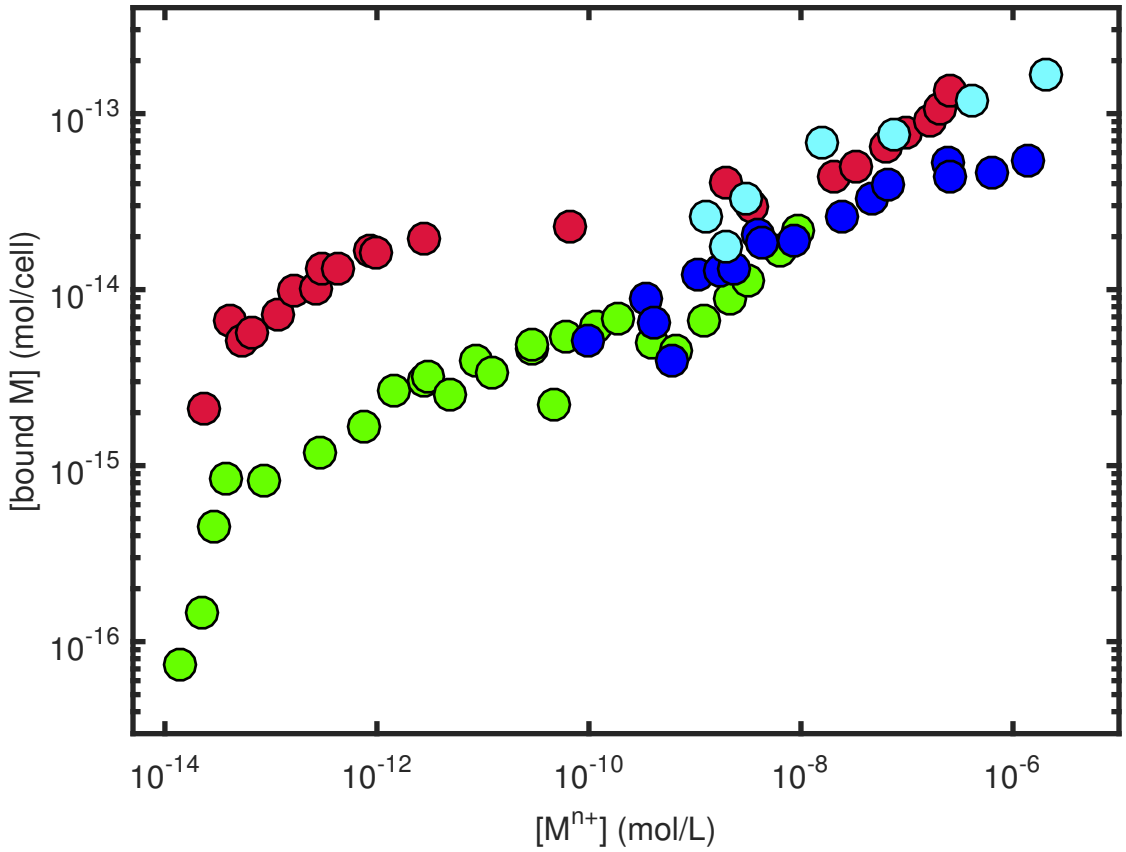

Figure 1: Cell suspension titration data, as measured free ion versus calculated bound metal per cell. Green (●) and red circles (●) are silver titrations of pavement (PVC) and mixed cell preparations (∼85% PVCs, ∼15% MRCs) respectively. Blue (●) and light blue (●) circles correspond to copper with pavement and mixed cell preparations respectively.

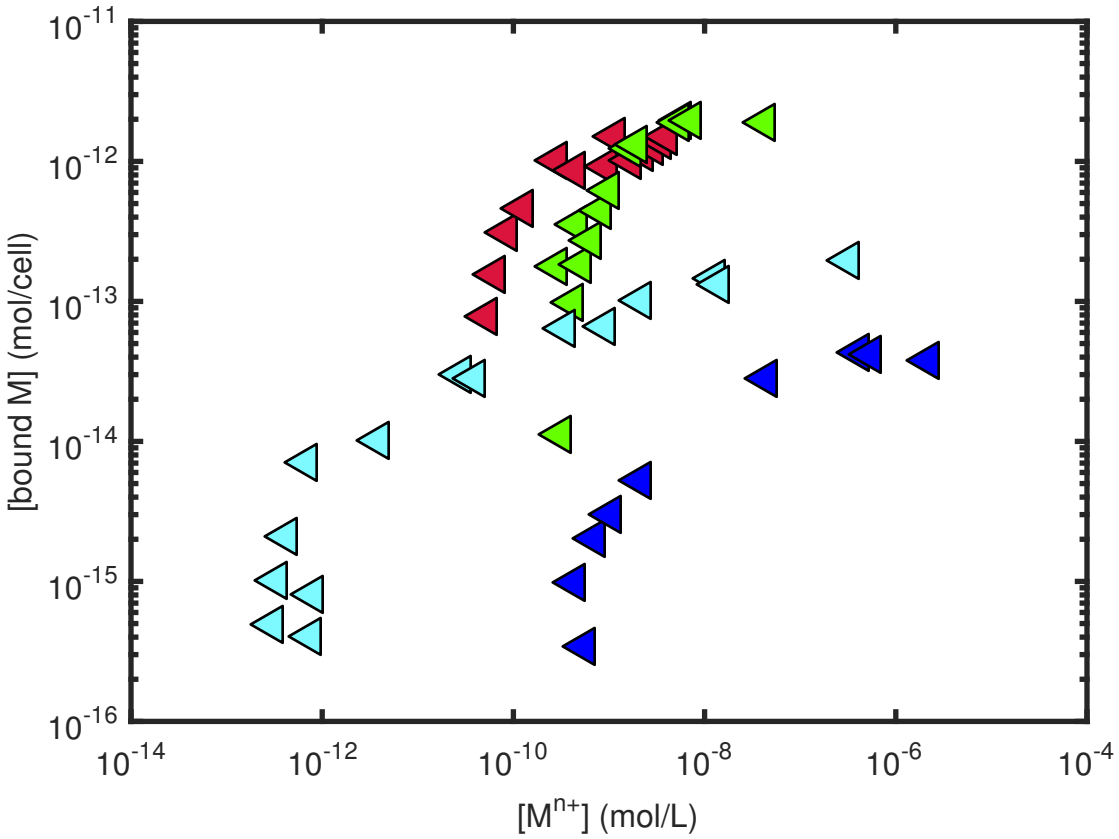

Figure 2: Titration data for reconstructed gill epithelia, as measured free ion versus calculated bound metal per cell. Green (▲) and red (▲) triangles are for silver with pavement (PVC) and mixed cell preparations (∼85% PVCs, ∼15% MRCs) respectively. Blue (▲) and light blue (▲) triangles correspond to copper with pavement and mixed cell preparations respectively.

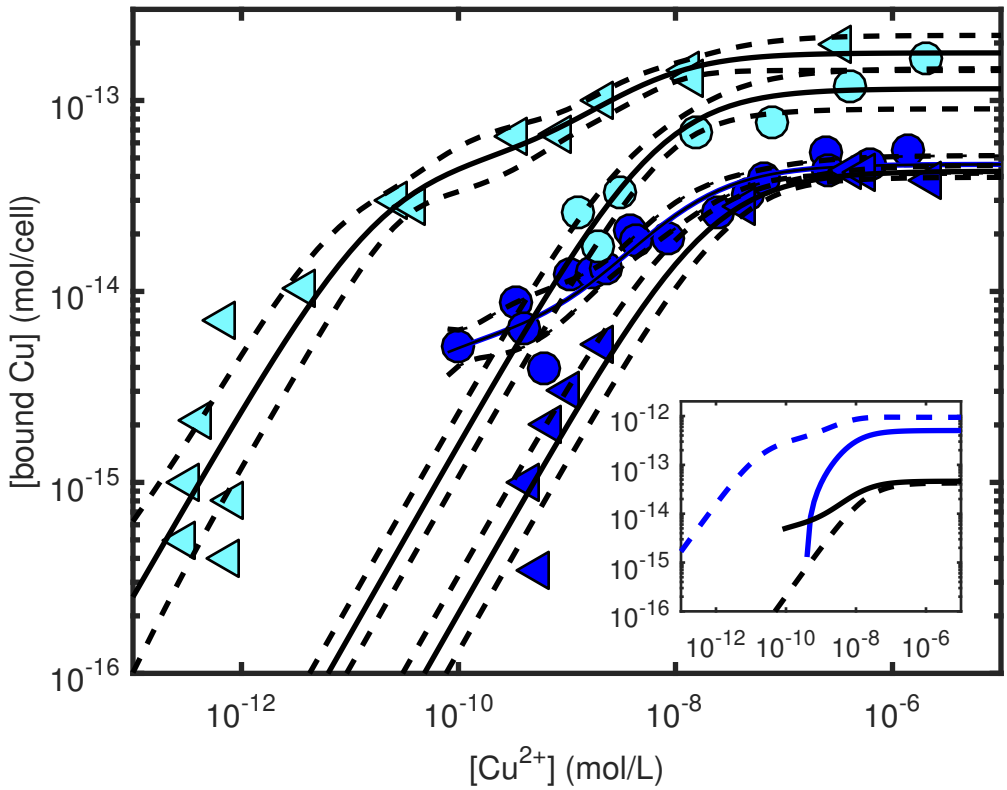

Figure 3: Copper titration curves displayed as measured free cupric ion versus calculated bound copper per cell. The dark blue symbols correspond to pavement cell (PVC) titrations, with the circles (●) representing cell suspension titrations and the triangles (▲) corresponding to reconstructed epithelium. The light blue symbols correspond to mixed cell titrations (∼85% PVCs, ∼15% MRCs) with circles (●) as suspensions and triangles (▲) corresponding to reconstructed epithelium. The solid lines correspond to model fits with dashed lines representing calculated 95% confidence intervals. The inset graph shows calculated MRC binding isotherms (see text) from epithelia data (dashed blue line) and from suspensions (solid blue line). The black lines show PVC alone binding isotherms for comparison.

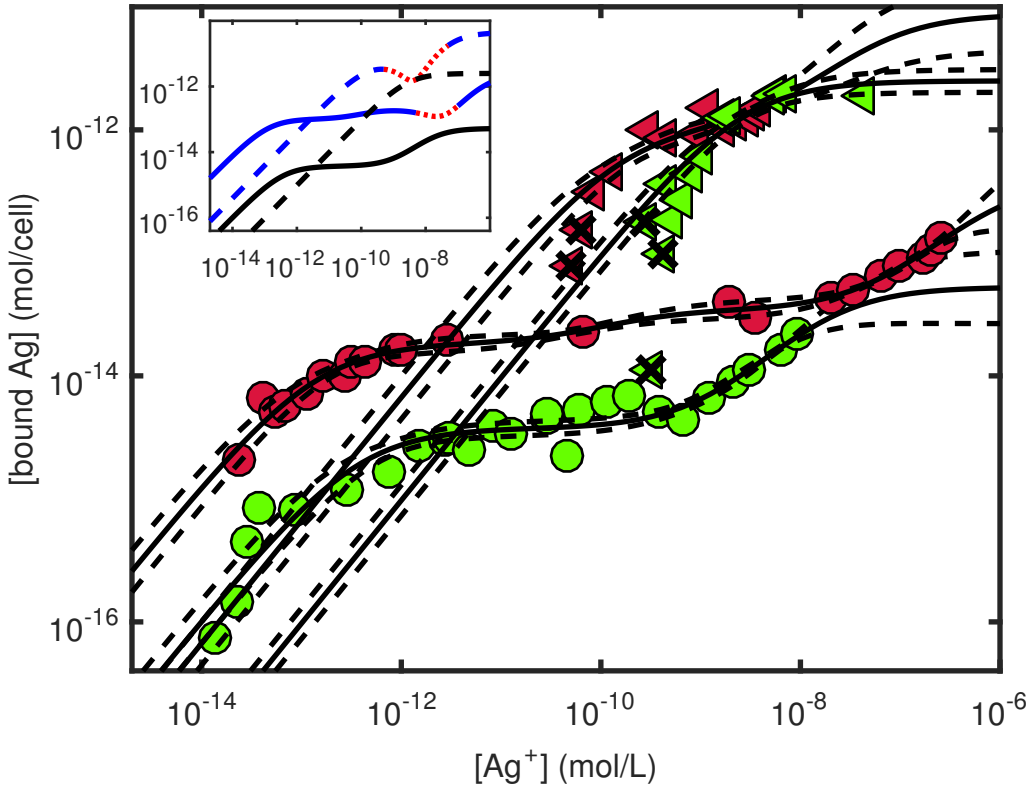

Figure 4: Silver titration curves displayed as measured free silver ion versus calculated bound silver per cell. The green symbols correspond to pavement cell (PVC) titrations, with the circles (●) representing cell suspension titrations and the triangles (▲) corresponding to reconstructed epithelia. The red symbols correspond to mixed cell tirations (∼85% PVCs, ∼15% MRCs), with circles (●) as suspensions and triangles (▲) corresponding to reconstructed epithelia. The solid lines correspond to model fits with dashed lines representing calculated 95% confidence intervals. The epithelium titration data points with X through them were not used in modelling. The inset graph shows calculated MRC binding isotherms (see text) from epithelia data (blue dashed line) and from suspensions (blue solid line); PVC alone data is shown for comparison as black lines. The regions indicated in red are not theoretically possible because the amount bound decreases as free ion increases).

Table 1: Parameter fitting results for an up to three site binding model to describe titration data. 95% confidence range of parameter estimates, determined by Monte Carlo analysis, are presented in italics under each parameter value. Binding capacities (L_T) values are presented in fmol/cell. [a]BLM parameter values are presented from the literature for comparison. For, the sample IDs, PVC-S=PVC suspensions, PVC-E=PVC epithelia, Mix-S=mixed cell suspensions, Mix-E=mixed cell epithelia. The values in blue were determined by fitting the MRC isotherms calculated from PVC and mixed cell titration data (MRC-S=MRC suspensions, MRC-E=MRC epithelia).

		Cu binding site		**Ag binding site**		
ID	**Parameter**	**1**	**2**	**1**	**2**	**3**
PVC-S	**log**K	11.1	8.0	12.4	9.1	
	logK range	*10.0-12.0*	*7.7-8.1*	*12.3-12.6*	*8.0-9.5*	
	$\mathbf{L}_T$	5.7	40.4	3.7	7.8	
	$\boldsymbol{L_T}$ ***range***	*4.3-8.9*	*37.7-43.0*	*3.4-4.1*	*4.8-35.5*	
PVC-E	**log**K		8.5		8.6	
	logK range		*7.7-8.9*		*8.5-8.7*	
	$\mathbf{L}_T$		41.9		2720	
	$\boldsymbol{L_T}$ ***range***		*33.7-66.2*		*3210-8300*	
Mix-S	**log**K		8.1	12.9	9.9	6.1
	logK range		*8.0-8.2*	*12.7-13.0*	*9.6-10.0*	*5.6-6.4*
	$\mathbf{L}_T$		115	18.2	16.0	377
	$\boldsymbol{L_T}$ ***range***		*100-131*	*16.8-19.9*	*12.3-19.8*	*217-931*
Mix-E	**log**K	10.8	8.5	9.8	8.2	
	logK range	*10.4-11.0*	*8.2-8.9*	*9.7-10.0*	*8.1-8.5*	
	$\mathbf{L}_T$	46.0	99.9	1020	7720	
	$\boldsymbol{L_T}$ ***range***	*33.8-66.2*	*17.3-141*	*870-1170*	*6730-8990*	
MRC-S	**log**K		7.8	12.9	10.2	5.8
	$\mathbf{L}_T$		549	102	72.4	2540
MRC-E	**log**K	10.7	8.6	10.0		7.1
	$\mathbf{L}_T$	304	651	4370		40,200
BLM[a]	**log**K		7.4,[2] 7.6[23]	10.0[6]	8.8[27]	mid 7 range[49]
	$\mathbf{L}_T$		1.2,[2] 0.8[23]	0.5[6]	0.001[27]	

Graphical TOC Entry