Dye-binding assay for the determination of sub-milligram quantities of suspended solids in freshwater [1]

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Summary. Clay and plankton particles from natural freshwater samples were collected on glass-fiber filters and stained with toluidine-blue to saturation at pH 8.6. The adsorbed dye was then eluted and quantified by spectrophotometry. The dye-binding capacity per dry weight was a consistent feature of the assemblage of particles contained in each sample, and varied very little between samples obtained from different water sources. This dye-binding assay is far more sensitive than the gravimetric determination of suspended solids.
Key words. Toluidine-blue; bacteria; phytoplankton; zooplankton; clays.

The use of biological dyes to quantify matter is widespread. Various dyes have been used in dye binding assays to quantify specific substances in solution, such as proteins [2] or acid polysaccharides [3]. Cationic dyes have been used for quantitative measurements of anionic groups in tissue sections [4] and for mass determination of bacteria adhering to membrane filters [5] or to polystyrene surfaces [6]. These methods for quantifying solids require adhesion to surfaces and quantification of the dye in its adsorbed state. Since any type of particle can be collected and stained on a support filter, and the adsorbed dye can then be desorbed (eluted) and quantified in solution, a general method for measuring suspended solids seemed feasable. The solids suspended in the water of lakes and ponds consist of planktonic organisms and of inanimate particles, predominantly clays. Concentration of total suspended solids is expressed as dry weight per volume, and its determination requires several milligrams of material per sample. In contrast, dye-binding assays are far more sensitive and could be used for the determination of suspended solids in the sub-milligram range. The aims of the present study were to select a dye which binds with no apparent specificity to particles encountered in freshwater samples, to characterize its staining reaction, and to choose the assay conditions which give reproducible dye-binding per unit dry weight within samples and the best uniformity among samples.

Materials and methods

Sampling and pretreatment of suspended solids. Grab water samples were collected from various sources in Israel and preserved in 1–4% formaldehyde. Zooplankton and phytoplankton were concentrated from waterbodies in Israel or in England with a hand-net with 60 µm pores and preserved in 4% formaldehyde. In the laboratory, these concentrates were size-fractionated by a series of small sieves and each size-fraction was homogenized using a Teflon homogenizer to yield fragments smaller than 60 µm. Dry weight (105 °C) and inorganic content (550 °C residue) were determined by standard methods [7].

Standard dye-binding assay. This protocol describes the assay conditions finally chosen, based on the experiments reported in the 'Results and discussion' section. All procedures were carried out at room temperature (20–23 °C). *Toluidine blue stock solution.* Toluidine blue O (Merck, lot 5281607) was dissolved to saturation in distilled water with 0.02% NaN_3. Dye concentration was monitored by absorbance measurements at 610 nm, after dilutions in 0.5% sodium dodecyl sulfate (SDS). The *staining solution* was prepared by diluting the stock solution (to yield 100–130 OD) into Tris-HCl buffer, pH 8.0, to a final concentration of 100 mM Tris. Dye addition raised the pH to 8.6. NaCl was added to a final concentration of 2 mM.

Assay. A suspension of particles (or fragments) in a syringe was forced through a GF/C filter (Whatman), 25 mm diameter, installed in a transparent filter holder (Schleicher and Schuell). Excess sample-liquid was forced out in order to eliminate dissolved substances which might affect the staining reaction. Prefiltered staining solution (see above) was then introduced into the top chamber of the holder, flooding the filter, and the holder was plugged at its outlet. After 30 min the assembly was washed by forcing 25 ml washing buffer (20 mM Tris-HCl, pH 8.6, with 2 mM NaCl) through it and was left full of buffer for 5 min. The assembly was washed again, emptied and placed over an elution vial. One ml of 2.5% SDS solution was added to the top chamber and the holder was plugged at the inlet. After 10 min the eluate was forced into the vial, and a further one ml of SDS solution was added and kept in the holder 10–20 min. Eight ml of water were then forced through the assembly into the elution vial, giving a total eluate volume of 10 ml (0.5% SDS). After mixing, the absorbance of the eluate was measured at 610 nm in a spectrophotometer (Gilford 300 N). Eluates with optical density higher than 1.6 were diluted in 0.5% SDS. Up to 30 samples were analyzed in each experiment; two blank filters were always included and gave a consistent background staining of 0.5–0.6 OD.

Calculations. The dye-binding capacity of the particles was expressed in toluidine-blue units (TBU). This unit reflects the number of dye-binding sites on the particles in the volume sampled, and hence it is a concentration unit parallel to mg l^{-1}.

1 TBU = $(OD_t - OD_b)\ 100 \times Ve/Vs$ where OD_t is the total absorption from loaded filters; OD_b, absorption from blank filters; Ve, volume of eluate (ml); Vs, volume of sample (ml). The B-value is the ratio TBU:mg l^{-1}, which reflects the number of dye-binding sites per mg (dry weight).

Results and discussion

Formaldehyde-preserved organisms, clay and other inanimate objects found in freshwater samples were all visibly stained by toluidine blue at neutral pH. Clays interacted with the dye immediately and formed aggregates. Dye penetration into metazoans was slow, but after homogenization their fragments were stained rapidly, as were intact algae and bacteria. After prolonged staining the entire matrix of each particle (with few exceptions) appeared to be evenly stained. Most of the dye remained bound after washing with water and could be desorbed by methanol or SDS. For quantitative analyses, glass-fiber filters of the type generally used for suspended solids determinations (GF/C) were employed to collect and handle the particles throughout the stages of staining, removal of excess dye, and elution of adsorbed dye. Figure 1 shows the amount of dye bound to blank filters or to filters carrying uniform aliquots of particles, at different pH values. Three samples of natural particle-assemblages were studied; two included only organic particles and the third contained 78% clay (Samples U, K, and B, respectively, in the table). The staining of blank filters was low up to pH 7.5 and rose as the pK of the dye (pH 9.6) was approached and the dye aggregated. The

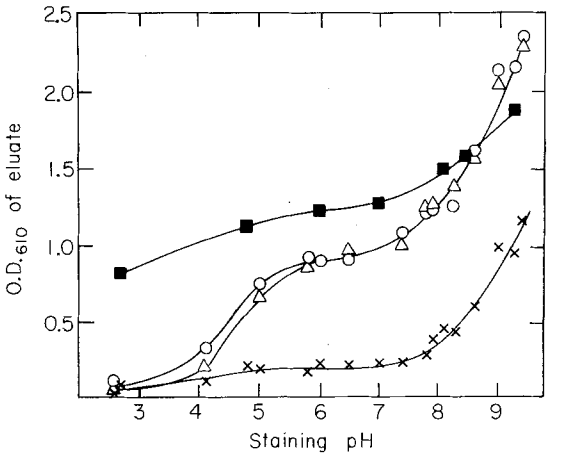

Figure 1. Adsorption of toluidine blue to particles at various pH values. Toluidine blue solution (130 OD) was buffered with 100 mM Tris-MES (and titrated with HCl if necessary) to the pH indicated. Excess dye was washed with 20 mM Tris-MES buffer at the same pH.
X, blank GF/C filters; ○, *Chlorella* sp. and bacteria from a sewage oxidation pond, 0.32 mg per filter; △, *Microcystis* sp., 0.39 mg per filter; ■, clay particles from a floodwater reservoir, 0.37 mg per filter. Each point shows the average of 2 determinations. Recorded values deviated from the average by 5% or less.

pH response of the two all-organic assemblages was identical: particle staining intensified at pH 5, was stable in the pH range of 5.5–7.5, and increased by 30% beyond pH 8.5. Net staining of inorganic particles was maximal over a wide pH range of 4.8–8.6. Since the staining reaction seemed to be least specific at pH. 8.6, this pH value was chosen for the standard procedure.

Studies of the time-course and dose-response of the staining reaction were made with the particles contained in

Table 1. Samples analyzed for dye-binding capacity per dry weight ("B-value")

Sample	Source, location and collection date		Size fraction (μm)	Composition	Percent inorganic +	B ± SEM	(n)
A	Rainwater puddle, Judean Mountains	Dec 1988	< 60	c,a,h,f	22	2.94 ± 0.07	(5)
B	Floodwater reservoir, Judean Mountains	Dec 1988	< 60	a,c,b	78	2.71 ± 0.10	(6)
C		Mar 1987	540–1000	q**	0	2.84 ± 0.24	(4)
D	Lake Windermere, Lake District*	Oct 1986	60–220	h,g	10	1.94 ± 0.27	(4)
E			> 220	p,m,r	3	2.81 ± 0.22	(6)
F	Drinking water reservoir, Lower Galilee	Oct 1986	> 60	m,p,r,ba	40	2.98 ± 0.10	(9)
G	Recreation lake, Odell Park, Beds.*	Oct 1986	> 60	p,r		3.14 ± 0.18	(4)
H	Recreation pond, Trent Park, London*	Oct 1986	60–220	k,h		2.84 ± 0.06	(4)
I			220–540	r,p		2.64 ± 0.12	(4)
J			> 540	s**	1.5	2.80 ± 0.14	(5)
K	Fish pool, Coastal Plain	Dec 1987	60–130	e**	0	2.86 ± 0.10	(10)
L	Irrigation water reservoir, Jezreel Valley	Jun 1987	> 60	r,k		2.63 ± 0.08	(4)
M	Fish pond, Beit She'an Valley	Dec 1988	< 60	c,b,a,h	25	3.20 ± 0.09	(4)
N	Effluent reservoir, Judean Plain	Aug 1987	60–130	m,k		3.26 ± 0.15	(5)
O			130–220	m**	1.9	3.27 ± 0.14	(6)
P			220–540	m,k	2.3	3.22 ± 0.05	(7)
Q	Effluent polishing pond, Coastal Plain	Jun 1987	60–110	j**	3	1.00 ± 0.08	(5)
R			130–220	o**	2.3	2.86 ± 0.11	(5)
S			> 1300	s**		2.76 ± 0.10	(7)
T	Sewage oxidation pond, Negev	Jan 1987	60–220	l**	0	2.80 ± 0.06	(6)
U	Sewage oxidation pond, Coastal Plain	Oct 1988	< 60	c,i,d	0	3.03 ± 0.09	(11)
V	Laboratory culture of *Saccharomyces cerevisiae*				0	3.13 ± 0.09	(10)

+ Inorganic content of < 5% fall within the error range of the determination method; n, Number of independently analyzed portions from each sample; * sampled in England; ** dominant organism (> 95% of mass).
a, clay; b, detritus; c, bacteria; d, *Thiocapsa* sp.; e, *Microcystis* sp.; f, ciliates; g, diatoms; h, chlorophytes; i, *Chlorella* sp.; j, *Pediastrum* sp.; k, rotifers; l, *Brachionus* sp.; m, cyclopoids; o, *Cyclops* sp.; p, calanoids; q, *Arctodiaptomus* sp.; r, cladocers; s, *Daphnia* sp.

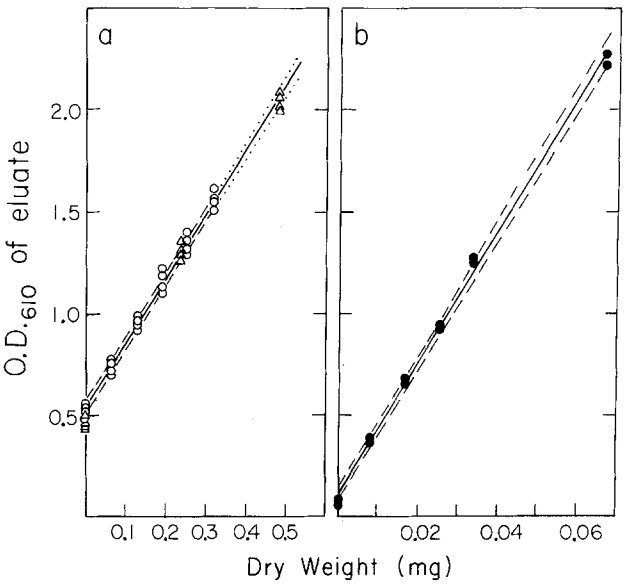

Figure 2. Extent of dye-binding as a function of sample mass.
a Standard assay (GF/C filters, 10 ml eluate). O, experiment no. 1, individual readings (n = 24); solid line, the calculated linear regression (OD = 0.53 + 3.10 × weight, r = 0.995, $p < 0.0001$), Coefficient of Variation = 3.3%; dashed line, 95% confidence interval of the means (C.I.M.); dotted line, predicted 95% C.I.M.; △, experiment no. 2, individual readings.
b Micro-assay (Eppendorf tubes, 1 ml eluate). Suspensions of particles derived from Sample U were centrifuged in Eppendorf tubes (10 min at 13 000 g) and the supernatants were discarded. Each pellet was resuspended in 0.2 ml staining solution, incubated for 30 min, washed twice with washing buffer, and resuspended in 0.2 ml 2.5% SDS. After 10 min, 0.8 ml water was added and the eluate was clarified by centrifugation. Other details were as described (Materials and methods). ●, individual readings (n = 12); solid line, the linear regression (OD = 0.12 + 31.4 × weight, r = 0.998, $p < 0.0001$), Coefficient of Variation = 4.3%; dashed line, 95% C.I.M.

Sample U. Dye binding was monitored as a function of staining duration (1–40 min) and dye concentration (16–400 OD, ca 0.03–0.7% w/v). The amount of dye adsorbed to the particles reached saturation within 20 min at 20 °C. When dye solutions of various concentrations were applied, the dye binding curve approached a plateau around 100 OD (not shown).

If all binding sites in a unit of mass are saturated, the amount of dye bound should be proportional to mass. This was tested by applying increasing volumes of a particle suspension (Sample U) to filters. The amount of dye adsorbed to each sub-sample was proportional to the volume applied, i.e. to the mass of suspended solids in it (fig. 2a). Linear calibration curves were obtained with quantities up to 0.5 mg per filter. The sensitivity of the standard assay (ca 0.1 mg, depending on the tolerated error) was limited due to high background staining of filters.

In a micro-assay, where Eppendorf tubes were employed instead of filters and the particles were separated from the various solutions by centrifugation, reduction of eluate volume and background staining enabled the determination of 0.005–0.07 mg with comparable precision (fig. 2b). The slope of the curves in figure 2 (b in the formula $y = a + bx$), which relates the dye-binding capacity of these particles to their dry weight, was defined as their 'B-value' (see Materials and methods).

The 'B-value' of various assemblages of particles (or their fragments) was analyzed by the standard assay in several independent determinations (table). This value appears to be a typical feature of each sample and can be determined with a reasonable reproducibility. For most samples the average 'B-value' fell within a narrow range of 2.95 ± 0.32 and was not related to the plankton composition. Furthermore, no correlation was found between the average 'B-value' and the inorganic content ($r = 0.034$). It is not surprising that the 'B-value' was a consistent characteristic of any one particle-assemblage, since all binding sites are expected to be saturated. On the other hand, the close similarity of the 'B-values' found for the different particle-assemblages, regardless of particle composition, season of collection, geographic location, or degree of water eutrofication, was unexpected. This finding can be related to the possible interactions between the dye and the plankton or clay particles exposed to it. The imidobase of toluidine blue is a planar hydrophobic molecule with a quarternary amine at the edge[4]. Several experiments which tested the effects of salts, acids, organic solvents and various detergents on staining or elution efficiency, indicated that the dye is adsorbed to organic matrices through cooperative electrostatic and hydrophobic interactions. Thus, if the dye interacts with all the major groups of biological macromolecules (as was observed), and if it can be partitioned into membranes and permeate other biological matrices, the average number of binding sites per unit of mass of organic matter could fall within a narrow range. The two exceptions encountered in this study (Samples D and Q in the table) contained algal assemblages which, as a result of intense selective predation, consisted mainly of algae possessing heavy and solid external structures, some with long appendages. These structures appeared unstained when observed under the microscope.

Clays of the smectite group bind aromatic dye cations both to the edge of the clay sheet (by ion exchange) and onto the interlayer space (by π bonds between the oxygen plane of the alumino-silicate on the surface of each sheet and the aromatic ring of the dye)[8]. It is possible that the smectite-rich clays found in Israel present a matrix of interlayer spaces which is similar to common organic matrices in the size of its components and/or in density of dye-binding sites. Other clays, such as kaolinite, do not adsorb dyes in the interlayer space[8] and therefore could give a different 'B-value', which might be related to particle size.

The variation in 'B-values', associated with certain phytoplankton assemblages or with clay composition, calls for caution in applying this quantification method to natural samples. Size-fractionation can be helpful in isolating exceptional assemblages from other plankton groups which can be determined with confidence.

This method of quantifying suspended solids is about 50 times as sensitive as the current weight determination method, and can reach the sensitivity range of protein determination[2] if the micro-assay is employed. It is apparently non-specific at high pH and can be applied, albeit with caution, to particles of diverse chemical composition. It is rapid, does not require sophisticated instruments and can be used in the field after a short exposure of the particles to formaldehyde. Once the ratio between the dye-binding capacity of a given particle-assemblage and their mass (the 'B-value') has been established, the method could be applied to many situations in microbiology, limnology, biotechnology or industry where sensitive quantification is required.

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Ansamitocin P-3, a maytansinoid, from *Claopodium crispifolium* and *Anomodon attenuatus* or associated actinomycetes[1, 2]

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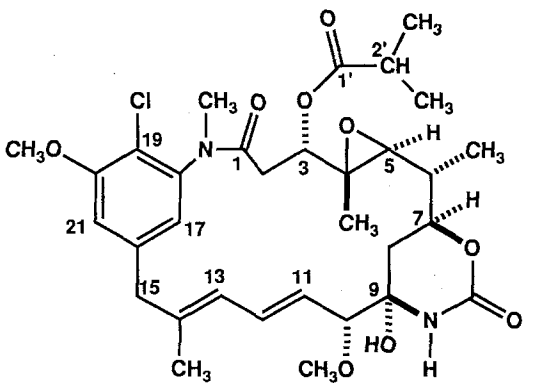
Summary. Guided by cytotoxicity, ansamitocin P-3, a maytansinoid, was isolated in very low yield from two members of the moss family Thuidiaceae, *Claopodium crispifolium* (Hook.) Ren. & Card. and *Anomodon attenuatus* (Hedw.) Hueb. Ansamitocin P-3 showed potent cytotoxicity against the human solid tumor cell lines A-549, HT-29. A possible basis for the occurrence of this compound in mosses is discussed.
Key words. Ansamitocin P-3; maytansinoids; *Claopodium crispifolium*; *Anomodon attenuatus*; Thuidiaceae; mosses; cytotoxicity; antitumor activity.

Previous screening for antitumor activity among the bryophytes (mosses, liverworts and hornworts) was established by cooperation between the US Department of Agriculture and the National Cancer Institute[4]. Among those species tested, two thuidiaceous mosses, *Claopodium crispifolium* (Hook.) Ren. & Card. and *Anomodon attenuatus* (Hedw.) Hueb. exhibited significant activity against P-388 lymphocytic leukemia in mice and both 9PS (murine lymphocytic leukemia) and 9KB (human nasopharyngeal carcinoma) cell culture systems and were, therefore, selected for further investigation to isolate the compounds responsible for the biological activity. In this communication, we report the cytotoxicity bioassay-directed isolation and identification of the active constituent, ansamitocin P-3 (**1**), from the active 10% aqueous methanol extracts of these two mosses.

C. crispifolium was collected from rocks on steep slopes in a Douglas fir forest in Oregon during May, 1981. The bioassay-guided fractionation of *C. crispifolium* is illustrated in the scheme. The whole air dried moss (13 kg) was ground and slowly percolated with 95% ethanol. The concentrated extract was partitioned between water and chloroform, and the concentrated chloroform extract was further partitioned between hexane and 10% aqueous methanol. The 10% aqueous methanol extract (49 g) showed cytotoxicity against 9PS and 9KB cells in culture at $ED_{50} = 3 \times 10^{-1}$ μg/ml and antitumor activity against the P-388 system in mice at %T/C = 147–



Ansamitocin P-3