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Morphological characterization of Libby "six-mix" amphiboles used in *in vivo* studies

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Abstract. — Shape and morphology were determined for 1,200 amphibole particles with width greater than 1 μm by use of the polarized light microscope (PLM) and for 1,200 particles with width less than 1 μm with a field emission electron microscope (FESEM). Also, for each particle the microscopist judged if the particle was a fiber or fragment based on its morphology. The amphiboles characterized herein were used in an *in vivo* experiment and are the so-called "Libby six-mix amphiboles": a mixture of six different samples collected by the USGS at the former Libby vermiculite mine site. For the particles greater than 1 μm they were determined to be 35% fibers, 46 % fragments, and 20% "not classified" (i.e., these particles could not with certainty be assigned to either class). For the particles less than 1 μm they were determined to be 34% fibers, 55% fragments, and 11% "not classified." However, if the aspect ratio, which is commonly used as a counting criteria to distinguish fibers from fragments is used, over 99% of the particles would be considers fibers. These results are in good agreement with an earlier study we conducted on a separate set of Libby amphibole samples.

Riassunto. — Sono state determinate la morfologia e le dimensioni di particelle di anfibolo: 1.200 particelle con larghezza superiore a 1 μm tramite microscopia a luce polarizzata (PLM) e 1.200 particelle con larghezza inferiore a 1 μm tramite microscopio elettronico in campo di emissione (FESEM). Inoltre, sulla base dell'osservazione microscopica, è stato possibile attribuire ad ogni particella l'appartenenza a "fibra" o a "frammento". Gli anfiboli caratterizzati in questo lavoro sono stati utilizzati per gli esperimenti *in vivo* e rappresentano la così detta "miscela di 6 anfiboli del Libby", una miscela di sei differenti campioni raccolti dall'USGS nei siti delle precedenti miniere di vermiculite del Libby. Per le particelle più grandi di 1μm, il 35% è stato classificato come fibre, il 46 % come frammenti e il 20% come "non classificate" (queste particelle potrebbero non avere una sicura assegnazione di classe). Le particelle inferiori a 1 μm sono risultate invece essere per il 34% fibre, il 55% frammenti e l'11% "non classificate". Comunque, utilizzando il rapporto morfologico comunemente usato per distinguere le fibre dai frammenti, oltre il 99% delle particelle dovrebbe essere considerato come fibre. Questi risultati sono in buon accordo con uno studio da noi condotto precedentemente su una serie separata di campioni di anfibolo provenienti dal Libby.

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Key words: *Libby, Montana, amphibole, amphibole morphology.*

Introduction

Animal studies are often used to serve as a model of the impact of inhalation of mineral dusts on humans. Critical to the success of these studies are both correct exposures of minerals and characterization of the mineral's morphology. The need for similar exposures is obvious, but the need for similar morphology is less so. Some health researchers believe that amphibole fibers are, in general, more harmful than fragments, while others believe there is no difference There also appears to be a correlation between the aspect ratio (i.e., the particle's length divided by its width) of a particle and its health impact - the higher aspect ratios appearing more harmful. (See Gunter *et al.*, 2007 and references therein for a more in-depth discussion of this on-going debate).

We undertook a study in collaboration with researchers from the University of Montana to aid them in determining the reproducibility of their mineral dose as well as its morphology. Their doses were on the order of micrograms and were prepared by a dilution of a known volume of solution and then instilled into mice lungs. The material they used was the so-called "Libby six mix" provided by the USGS (United States Geological Survey). The Libby six mix derives its name because it is a mixture of six of the 30 amphibole sampling locations collected by the USGS from the now closed vermiculite mine near Libby, Montana in 2000; they are sample numbers 20, 23, 25, 27, 28, and 30 from Meeker *et al.* (2003).

Another reason we undertook this study was the amphiboles occurring in the former vermiculite mine near Libby, Montana have been related to negative health effects in the miners who worked at that deposit (see Bandli and Gunter, 2006, and references therein). The amphiboles from that deposit have also been chemically, structurally, optically, and morphologically characterized in several studies (Bandli, *et al.*, 2003; Brown and Gunter, 2003; Gunter *et al.*, 2003; Meeker *et al.*, 2003). And while some of the amphiboles at the deposit are asbestiform, others are not as pointed out in the above studies. This is important because there is still an ongoing debate about the possible differing health effects of fibers vs. fragments as stated above. Regardless, currently in the United States only the asbestiform amphiboles are regulated (OSHA, 1992), and then only five amphibole species. Last these same samples have recently been used in *in vitro* experiments (Hillegass *et al.*, 2008), and showed a lower toxic effect than crocidolite in gene profiling experiments on human mesothelial cells.

Methods

We were provided with two sample sets (labeled "S" and "P") of the Libby six mix deposited on duplicate filters (labeled S1 & S2 and P1 & P2) for a total of four total filters; the mineral/solution formed an approximate 6 mm spot on each of the four filters (upper row of photographs in Fig. 1). The samples were prepared by placing a known amount of amphibole into a known volume of saline solution in a ratio of 30 μL solution to 100 μg amphibole. This mixture was sonicated for four minutes in an eppendorf dropper and 150 μL was spotted on each of the four mixed cellulose filters. This was the same method use to administer the dose in the *in vivo* experiments.

Two separate microscopic methods were used to measure the particle's width and length and to determine, based on morphology, if the particles were fibers or fragments; the PLM (polarizing light microscope) was used for particles greater than one micron in width and the FESEM (field emission scanning electron microscope) was used for particles less than one micron in width. A total of 2400 particles - 1200 from the PLM and 1200 from the FESEM, and consisting of 300 particles from each of the four filters - were measured and then classified as fibers or fragments based on morphology or as a fiber if the particle's aspect ratio was greater than 3:1.

Results and Discussion

Tables 1-4 list the results for particles measured in the PLM (i.e., particles > 1 μm in diameter), while tables 5-8 list the results for the FESEM (i.e., particles < 1 μm in diameter). Tables 1 and 5 give the number of particles placed in different width and length categories. Tables 2 and 6 list the percent of fibers, fragments, and "not classified" (as determined by the microscopist based on

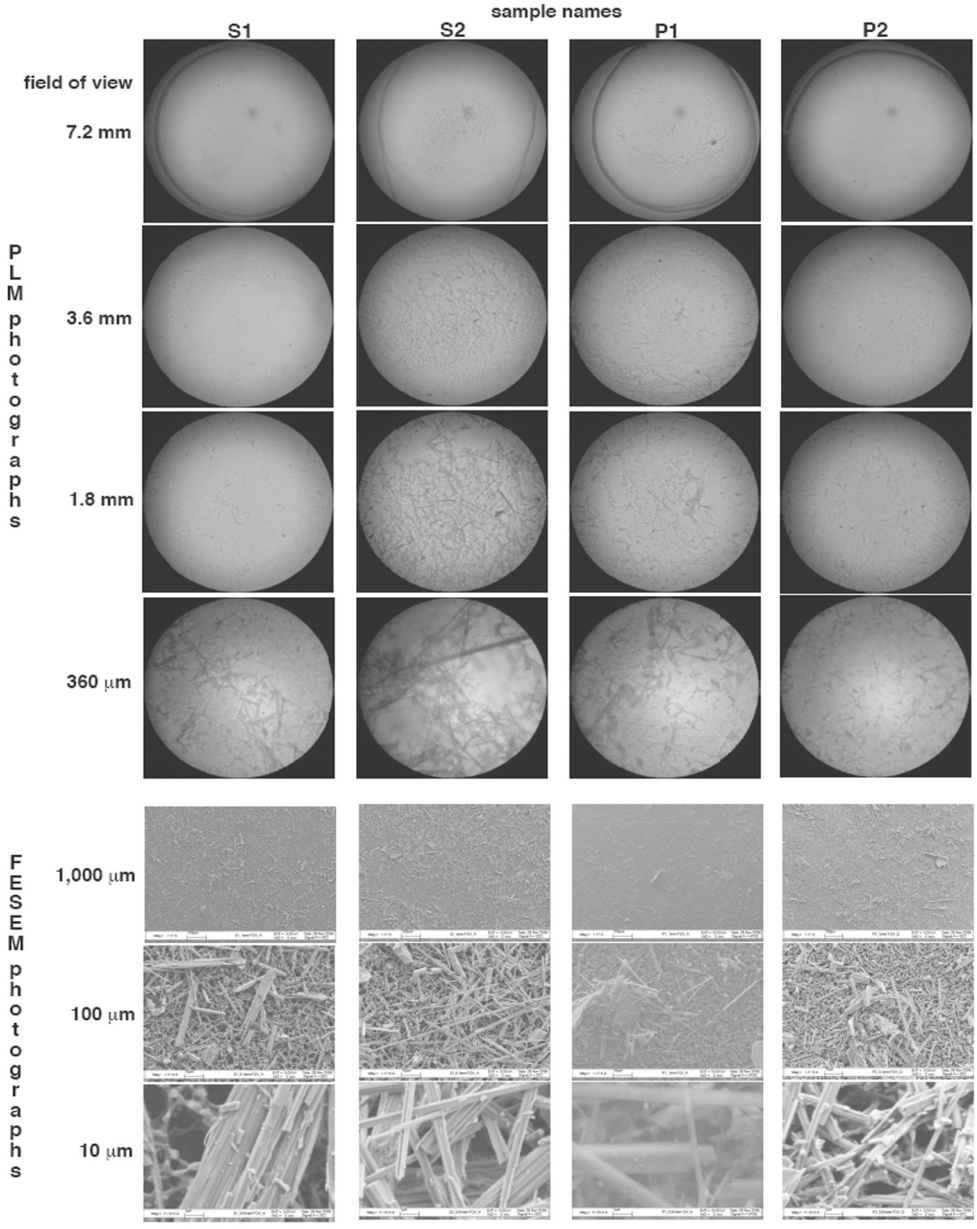

Fig. 1 – Photographs of amphibole particles on filters. The four columns represent our four separate samples: S1 and S2 are from the same split and P1 and P2 are from a different split. The upper four rows are photographs taken with the PLM, while the lower three are taken with the FESEM. The field of view for each row is given to its left; as can be seen the images increase in magnification from the top to bottom of the figure. Based on these photos the doses are not the same and the particles size would exceed the respirable size. Also, note than many of the particles do not exhibit a fibrous morphology.

Table 1
Size distribution (by particle) for S1, S2, P1, and P2 as determined with the PLM

sample	width (μm)	length (μm)				
		0-10	11-20	21-50	51-100	>100
S1	0-1	0	4	20	10	1
(n=300)	1.1-2	1	11	56	24	1
	2.1-5	2	22	74	25	6
	5.1-10	0	8	18	7	4
	>10	0	0	1	3	2
S2	0-1	0	3	21	11	4
(n=300)	1.1-2	1	13	55	23	8
	2.1-5	0	18	69	29	13
	5.1-10	0	1	9	14	8
	>10	0	0	0	0	0
P1	0-1	0	8	8	4	0
(n=300)	1.1-2	4	19	51	15	4
	2.1-5	0	26	71	26	7
	5.1-10	0	3	23	19	4
	>10	0	0	1	2	5
P2	0-1	0	5	15	5	1
(n=300)	1.1-2	3	15	49	19	2
	2.1-5	3	33	71	21	5
	5.1-10	1	7	21	16	3
	>10	0	0	2	2	1

morphology) as a function of seven aspect ratio classes. Tables 3 and 7 were assembled to help determine if the two different splits of the six-mix (herein labeled "S" and "P") are similar. Last, Tables 4 and 8 summarize the classification of the particles based on aspect ratio and morphology.

Fig. 1 shows photographs of the four samples with increasing magnifications. Notice that the amount of material on the four filters appears to differ; this would indicate the dose would differ,

Table 2
Percent of fibers, fragments, and not classified particles in the S1, S2, P1, and P2 determined morphologically and grouped by aspect ratio (l/w)

sample	aspect ratio	fibers (%)	fragments (%)	not classified (%)	total (%)
S1	<3	0	0.67	0.67	1.33
	3-5	1.33	7.00	5.00	13.33
	6-10	7.67	10.67	6.33	24.67
	11-20	12.67	10.33	10.33	33.33
	21-50	9.67	4.67	9.00	23.33
	51-100	2.67	0	1.00	3.67
	>100	0.33	0	0	0.33
S2	<3	0	0.33	0	0.33
	3-5	1.33	4.33	1.00	6.67
	6-10	4.67	14.33	3.00	22.00
	11-20	13.33	19.00	5.33	37.67
	21-50	12.00	6.00	8.33	26.00
	51-100	4.67	1.00	0.33	6.00
	>100	1.00	0	0.33	1.33
P1	<3	0	0.33	0	0.33
	3-5	1.00	12.67	1.00	14.67
	6-10	7.67	21.67	4.67	34.00
	11-20	12.33	10.67	6.00	29.00
	21-50	10.67	6.00	2.33	19.00
	51-100	1.67	0.00	1.00	2.67
	>100	0.33	0	0	0.33
P2	<3	0	2.00	0	2.00
	3-5	0.33	17.67	1.00	19.00
	6-10	4.67	18.00	3.00	25.67
	11-20	13.33	11.00	6.00	30.33
	21-50	12.67	4.33	3.67	20.67
	51-100	2.00	0	0	2.00
	>100	0.33	0	0	0.33

TABLE 3a
Percent of fibers, fragments, and not classified particles in the S samples combined from Table 2, and grouped by aspect ratio (l/w)

aspect ratio	fibers (%)	fragments (%)	not classified (%)
<3	0	0.50	0.33
3-5	1.33	5.67	3.00
6-10	6.17	12.50	4.67
11-20	13.00	14.67	7.83
21-50	10.83	5.33	8.67
51-100	3.67	0.50	0.67
>100	0.67	0	0.17

Table 3b
Percent of fibers, fragments, and not classified particles in the P samples combined from Table 2, and grouped by aspect ratio (l/w)

aspect ratio	fibers (%)	fragments (%)	not classified (%)
<3	0	1.17	0
3-5	0.67	15.17	1.00
6-10	6.17	19.83	3.83
11-20	12.83	10.83	6.00
21-50	11.67	5.17	3.00
51-100	1.83	0	0.50
>100	0.33	0	0

TABLE 4
Summary of classification of fibers, fragments, and not classified for the four samples based on aspect ratio and morphology as determined in the PLM

	sample	fibers (%)	fragments (%)	not classified (%)
A. aspect ratio	S1	98.67	1.33	-
	S2	99.67	0.33	-
	P1	99.67	0.33	-
	P2	98.00	2.00	-
B. morphology	S1	34.33	33.33	32.33
	S2	37.00	45.00	18.33
	P1	33.67	51.33	15.00
	P2	33.33	53.00	13.67

TABLE 5
Size distribution (by particle) for S1, S2, P1, and P2 as determined with the FESEM

sample	width (μm)	length (μm)				
		0-5	5.1-10	10.1-20	20.1-30	>31
S1	0-0.2	9	15	8	0	0
(n=300)	0.21-0.4	27	65	41	7	4
	0.41-0.6	10	22	19	6	0
	0.61-0.8	14	15	15	2	2
	0.81-1.0	4	8	5	2	0
S2	0-0.2	3	20	7	0	0
(n=300)	0.21-0.4	29	82	46	6	6
	0.41-0.6	4	24	13	4	1
	0.61-0.8	10	18	14	3	5
	0.81-1.0	1	1	2	1	0
P1	0-0.2	13	27	6	1	0
(n=300)	0.21-0.4	48	74	28	3	0
	0.41-0.6	5	45	13	2	0
	0.61-0.8	3	15	8	2	1
	0.81-1.0	2	2	2	0	0
P2	0-0.2	7	27	10	0	0
(n=300)	0.21-0.4	30	87	47	6	1
	0.41-0.6	5	23	15	3	2
	0.61-0.8	4	13	11	2	0
	0.81-1.0	2	3	2	0	0

which would cause difficulty in interpreting the results of the animal study. Also, many of the amphibole particles are too large to be respirable.

To describe the morphology we used two methods to distinguish fibers vs. fragments, one is the aspect ratio which is used as a counting statistic and the other is morphology (i.e., does the particle appear as a fiber or fragment). Based on the aspect ratio, 99% of the particles were fibers. Based on

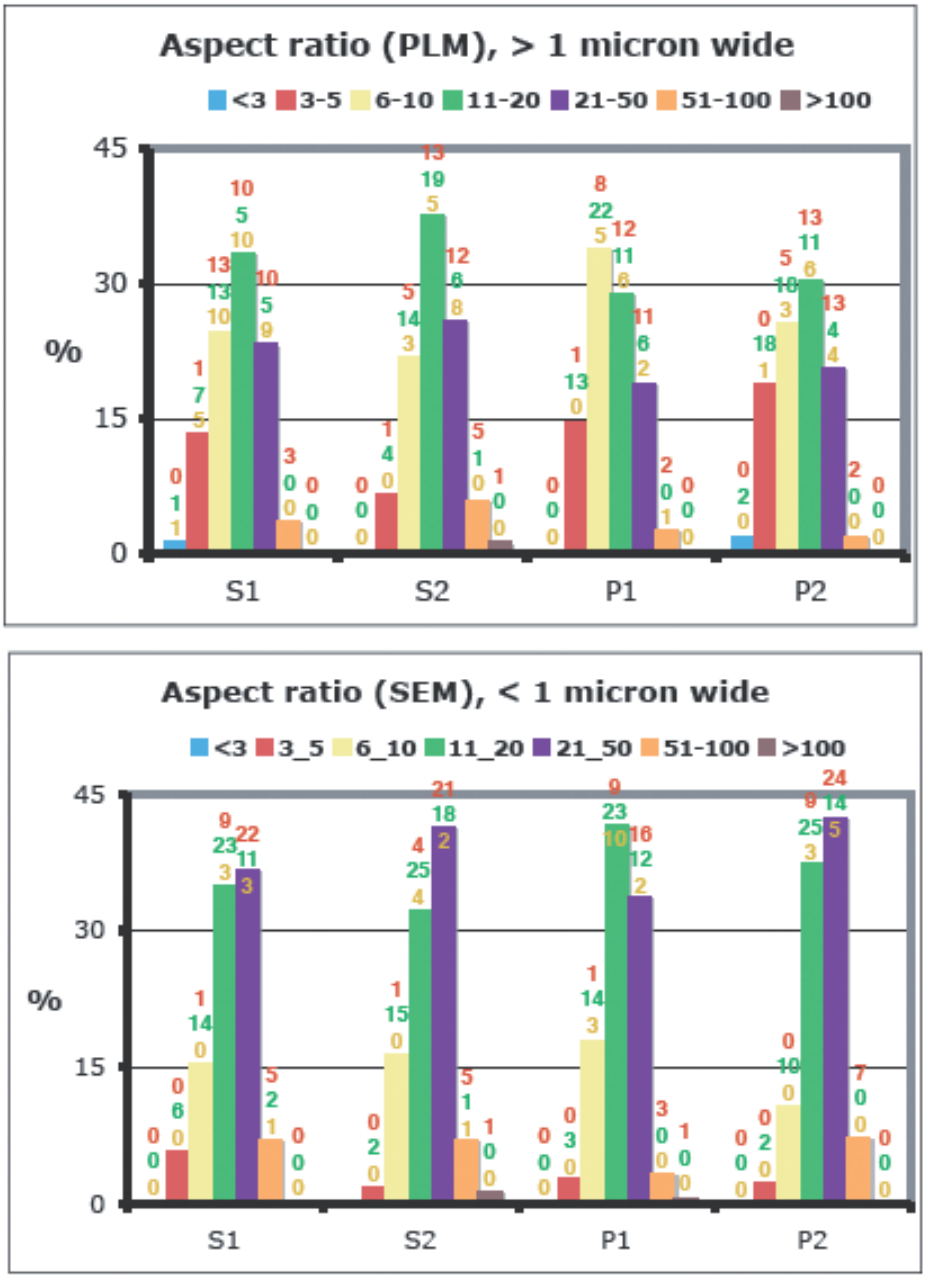

Fig. 2 – The upper bar graph shows the distribution of aspect ratios for particles greater than 1 μm and the bottom graph for those less than 1 μm. The three numbers above each bar represents the overall percent of fibers (in red), fragments (in green), and indistinguishable particles (in yellow) for that sample (rounded to the nearest whole number). Note that all four distributions appear somewhat similar with the majority of the particles falling in the 11-20 and 21-50 aspect ratio ranges. More interestingly note that, in general, the microscopist determined the smaller aspect ratio were fragments, the longer ones were fibers, and the intermediate aspect ratios were difficult to classify as either fragments of fibers.

TABLE 6
Percent of fibers, fragments, and not classified particles in S1, S2, P1, and P2 determined morphologically and grouped by aspect ratio (l/w)

sample	aspect ratio	fibers (%)	acicular (%)	not classified (%)	total (%)
S1	<3	0	0.33	0	0.33
	3-5	0	5.67	0	5.67
	6-10	1.00	14.00	0.33	15.33
	11-20	8.67	23.33	3.00	35.00
	21-50	22.33	11.33	3.00	36.66
	51-100	4.67	1.67	0.67	7.00
	>100	0	0	0	0
S2	<3	0	0	0	0
	3-5	0	1.67	0	1.67
	6-10	0.67	15.33	0.33	16.33
	11-20	3.67	24.67	4.00	32.34
	21-50	20.67	18.33	2.33	41.33
	51-100	4.67	1.33	1.00	7.00
	>100	1.33	0	0	1.33
P1	<3	0	0	0	0
	3-5	0	2.67	0	2.67
	6-10	0.67	14.33	3.00	18.00
	11-20	8.67	22.67	10.33	41.67
	21-50	15.67	12.00	6.00	33.67
	51-100	2.67	0.33	0.33	3.33
	>100	0.67	0	0	0.67
P2	<3	0	0	0	0
	3-5	0	2.33	0	2.33
	6-10	0	10.33	0.33	10.66
	11-20	9.00	25.00	3.33	37.33
	21-50	23.67	14.00	4.67	42.34
	51-100	7.00	0.33	0	7.33
	>100	0	0	0	0

TABLE 7a
Percent of fibers, fragments, and not classified particles in the S samples combined from Table 2, and grouped by aspect ratio (l/w)

aspect ratio	fibers (%)	acicular (%)	not classified (%)
<3	0	0.17	0.17
3-5	0	3.67	3.67
6-10	0.83	14.67	15.83
11-20	6.17	24.00	33.67
21-50	21.5	14.83	39.00
51-100	4.67	1.50	7.00
>100	0.67	0	0.67

TABLE 7b
Percent of fibers, fragments, and not classified particles in the P samples combined from Table 2, and grouped by aspect ratio (l/w)

aspect ratio	fibers (%)	acicular (%)	not classified (%)
<3	0	0	0
3-5	0	2.50	0
6-10	0.33	12.33	1.67
11-20	8.83	23.83	6.83
21-50	19.67	13.00	5.33
51-100	4.83	0.33	0.17
>100	0.33	0	0

morphology, 34% were fibers, 46% fragments, and 20% could not be differentiated by the PLM; 34% were fibers, 55% fragments, and 11% could not be differentiated by the FESEM. More specifically, we found particles with low aspect ratios morphologically had a higher percentage of fragments, particles with high aspect ratios morphologically had a higher percentage of fibers, and particles with intermediate aspect ratios were difficult to classify as either fibers or fragments based on morphology. These trends can be seen in Fig. 2, which shows bar graphs for the four samples (i.e., S1, S2, P1, and P2), where the upper

Table 8
Summary of classification of fibers, fragments, and not classified for the four samples based on aspect ratio and morphology with FESEM data

	sample	fibers (%)	fragments (%)	not classified (%)
A. aspect ratio	S1	99.67	0.33	-
	S2	100	0	-
	P1	100	0	-
	P2	100	0	-
B. morphology	S1	36.67	56.33	7.00
	S2	31.00	61.33	7.67
	P1	28.33	52.00	19.67
	P2	41.67	52.00	8.33

graph is for particles > 1 μm in width and the lower one < 1μm in width. Also, atop each bar are three numbers, where the top one is the percent fibers, the middle one the percent of fragments, and the lower one the number of "not classified" (note these percentages are rounded to the nearest whole number for ease of displaying).

Brown and Gunter (2003) performed a similar set of optical characterizations using the PLM on a suite of three samples collected at the former Libby vermiculite mine by Gunter in 1999. Photographs of these samples are shown, along with compositional, optical, and morphological data in Bandli *et al.* (2003). Interestingly, Brown and Gunter (2003) obtained similar results on that sample set as found herein on the "six-mix." Based on the aspect ratio they found that 95% of the particles were fibers and based on morphology they found 36% fibers, 33% fragments, and 31% they could not differentiate. Thus the amphiboles occurring at the Libby deposit are approximately 50/50 fibers and fragments based on morphology, while almost all of the particles would meet the counting rule to be considered a fiber. Clearly this significant discrepancy presents an issue in attempting to classify these particles.

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