

1 **Inhalable textile microplastic fibers impair airway epithelial growth**

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3 **Short title: Textile microplastics impair organoid growth**

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32 **Abstract (150 words)**

33 We are continually exposed to microplastic fibers originating from textiles. High exposure  
34 to microplastic fibers in nylon flock workers has been linked to the development of airway  
35 and interstitial lung disease, but the exact health effects of microplastic fibers on the lungs  
36 are unknown. Here we determined effects of polyester and nylon textile microplastic fibers  
37 on airway and alveolar epithelial cells using human and murine lung organoids. We  
38 observed that the presence of particularly nylon microfibers had a negative impact on the  
39 growth and development of airway organoids. This effect was mediated by components  
40 leaking from nylon. Moreover, our data suggested that microplastic textile fibers may  
41 especially harm the developing airways or airways undergoing repair. Our results call for an  
42 urgent need to assess exposure and inhalation levels in indoor environments to accurately  
43 determine the actual risk of these fibers to human health.

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## 46 **Introduction**

47 Plastic pollution is a pressing global concern and microplastics are a significant part of this  
48 problem (1). High amounts of microplastics have been found in marine environments, air,  
49 soils, plants, and animals, which illustrates how omnipresent this relatively recent pollution  
50 actually is (2). Microplastic pollution derives from personal care products, synthetic clothes,  
51 and degradation of macroplastics (3, 4). Synthetic textile fibers are one of the most  
52 prevalent types of microplastic waste observed, with an annual production of 60 million  
53 metric tons, which equals 16% of the world's plastic pollution (1). These fibers are typically  
54 composed of nylon or polyester and are released into the environment by wear and tear and  
55 during washing and drying of garments (4-6).

56 The ubiquitous nature of microplastics in the environment inevitably leads to human  
57 exposure, which can occur through two main routes (7, 8). Firstly, through ingestion of  
58 contaminated food and water and secondly via inhalation. Microplastics have been reported  
59 both in indoor and outdoor air, with levels indoors being 2-5 times higher as compared to  
60 outdoors (9, 10). Whether or not microfibers can deposit in lung tissue largely depends on  
61 the aerodynamic diameter of the fibers (7, 11). Lung deposition is most efficiently achieved  
62 with aerodynamic diameters between 1-10  $\mu\text{m}$  (12), however, these sizes are difficult to  
63 quantify in environmental samples due to limitations of the analytical techniques (9, 13).  
64 Yet, plastic microfibers have been found in human lung tissue, suggesting inhalation does  
65 indeed take place (14). Furthermore, several studies from workers in synthetic textile, flock  
66 and (poly)vinyl chloride industries suggest that inhalation of such microfibers is harmful, as  
67 around 30% of factory workers developed work-related airway and interstitial lung disease  
68 (15-23). Moreover, exposure to particulate matter in air pollution, also containing  
69 microplastics (24-26), has been associated with higher risk of developing asthma and an  
70 increase in asthma symptoms in areas with higher levels of particulate matter air pollution  
71 (27-29).

72 Despite the potential capacity for microplastic fibers to contribute to respiratory diseases,  
73 the health effects are greatly understudied and information providing evidence of potential  
74 human health effects of inhaled microplastics is lacking (9, 30, 31). In the present study, we  
75 therefore explored whether textile microplastic fibers can cause damage to lung tissue. As  
76 epithelial cells are the first to come into contact with inhaled fibers, we investigated effects  
77 of polyester and nylon microfibers on lung epithelial proliferation, differentiation, and

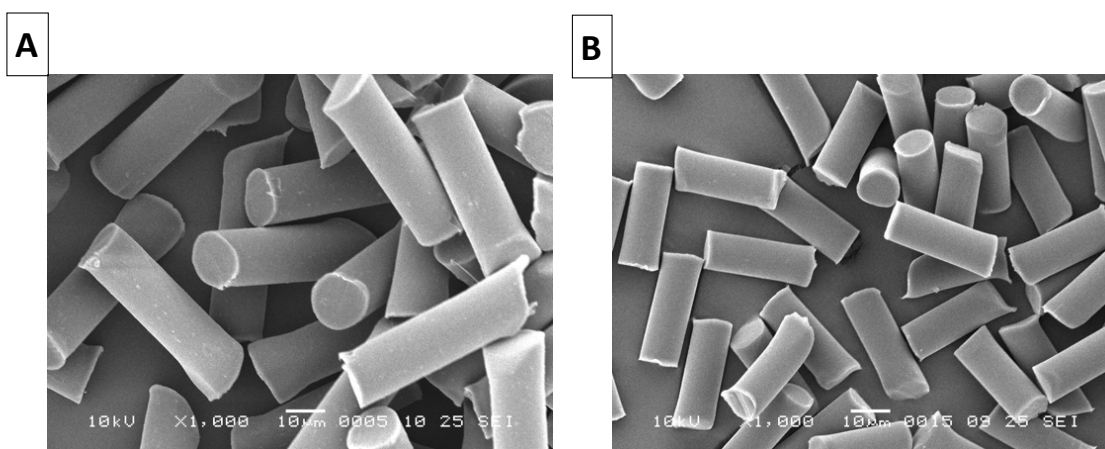
78 repair processes. For this we used lung organoids that are grown from primary lung  
79 epithelial progenitor cells with support of a lung fibroblast cell line (32, 33). The epithelial  
80 progenitors can develop into organoids consisting of alveolar epithelial cells or organoids  
81 consisting of airway epithelial cells. We found that in particular nylon microfibers  
82 negatively impacted developing airway organoids, while developing alveolar organoids and  
83 already developed organoids of both types appeared to be less affected. This negative effect  
84 was caused by still unknown leachates from nylon that particularly inhibit differentiation of  
85 airway epithelial cells. We therefore urge for assessment of exposure levels in indoor  
86 environments and actual lung deposition to accurately determine the risk of these fibers to  
87 human health.

88

## 89 Results

### 90 Characterization of reference microfibers

91 To produce reference textile fibers, we used a method previous described by us to  
92 reproducibly generate fibers of specific lengths (34). Fibers are commonly defined as  
93 having a length to diameter ratio of 3:1 (35). The smallest polyester diameter commercially  
94 available is 15  $\mu\text{m}$ , while nylon is available in 10  $\mu\text{m}$  diameters, therefore the smallest  
95 fibers we could produce were 45 and 30  $\mu\text{m}$  for polyester and nylon respectively. The fibers  
96 we produced had a median size of 15x52  $\mu\text{m}$  for polyester and 12x31  $\mu\text{m}$  for nylon (Table  
97 S1). They were rod-shaped with a circular cross-section and had a smooth surface, as shown  
98 with scanning electron microscopy (SEM) (Figure 1A and B). For our polyester fibers  
99 energy dispersive X-ray (EDX) analysis confirmed the presence of carbon and oxygen  
00 (Figure S1A). The recorded micro-Fourier transform infrared ( $\mu\text{FTIR}$ ) spectrum showed  
01 characteristic absorbance peaks of polyester (Figure S1B). The EDX spectrum for nylon  
02 confirmed the presence of carbon, nitrogen and oxygen (Figure S1C) and the  $\mu\text{FTIR}$   
03 spectrum showed characteristic nylon absorbance peaks (Figure S1D).



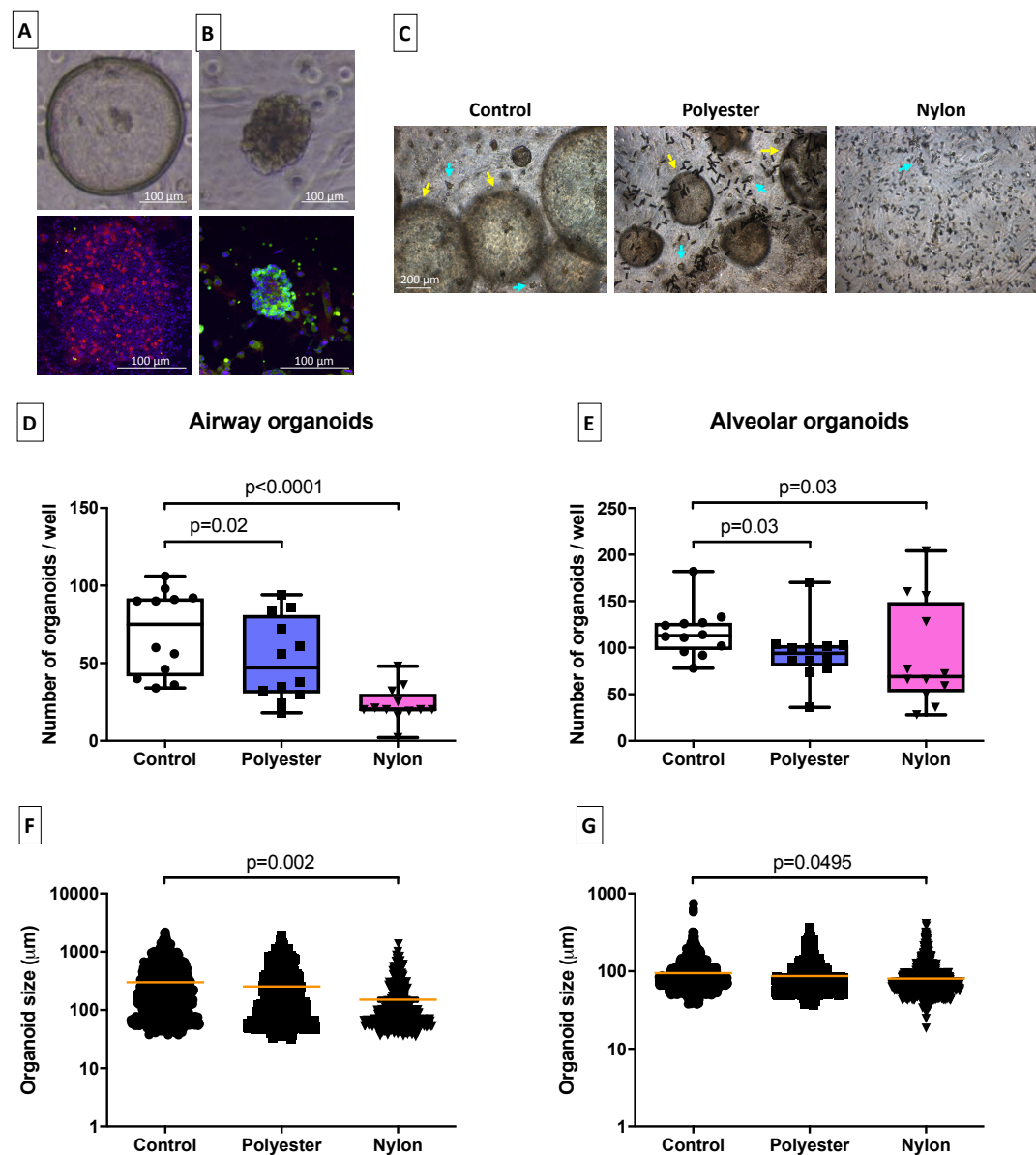
04  
05 **Figure 1. Morphology of reference microplastic fibers of standardized dimensions.**  
06 *Representative SEM micrographs of (A) polyester microfibers (15x52  $\mu\text{m}$ ) and (B) nylon*  
07 *microfibers (12x31  $\mu\text{m}$ ).*

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## 10 **Nylon microfibers inhibited growth of murine and human lung organoids**

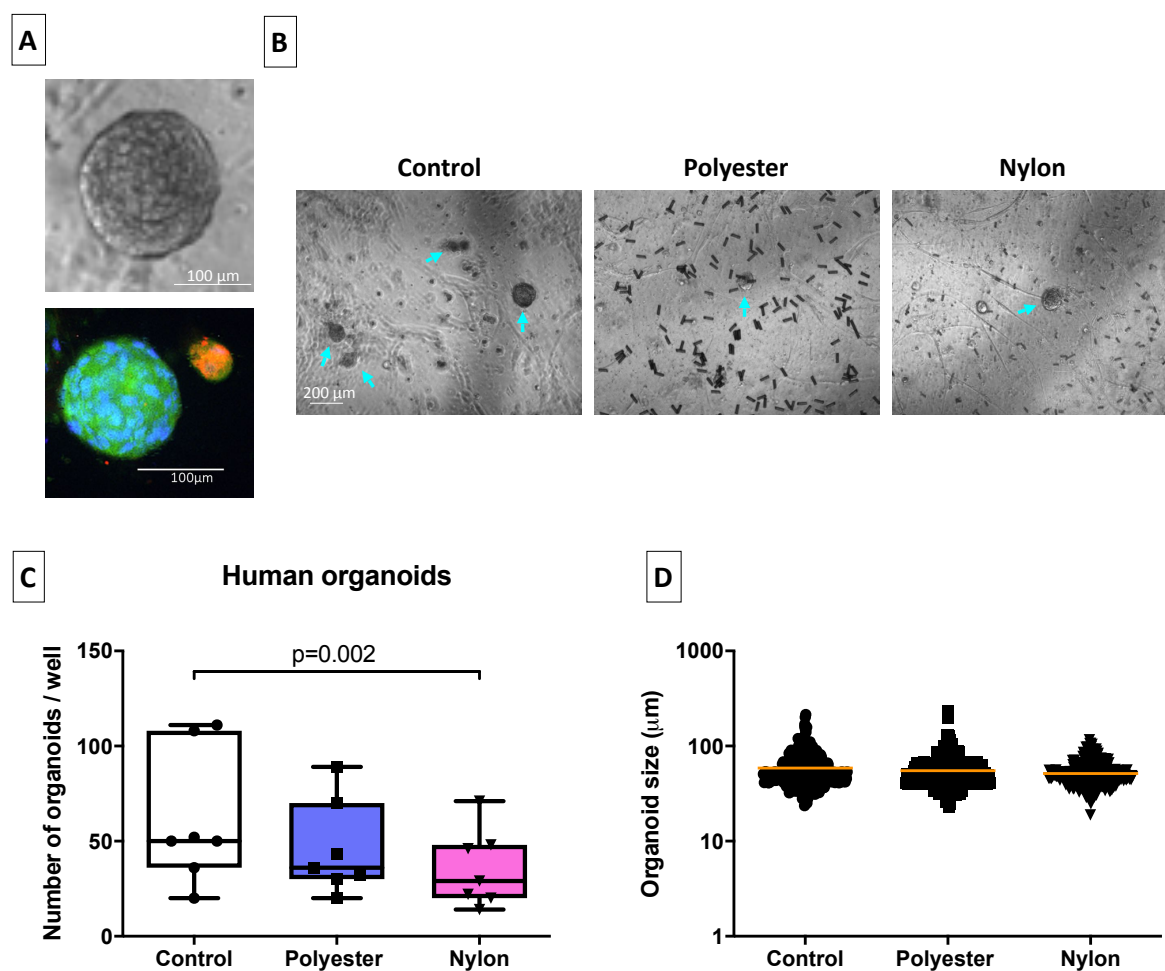
11 Possible effects of microplastic fibers on lung epithelial proliferation, differentiation, and  
12 repair processes were assessed *in vitro* using both murine and a human lung organoids. For  
13 this, we first assessed the effects of several of several doses of fibers on organoid growth  
14 ranging from 2000-5000 fibers per well (Figure S2). The fibers were dispersed into the  
15 matrigel in which the organoids grow at the same time as the isolated epithelial cells and  
16 fibroblasts were added. We decided to continue with 5000 polyester or 5000 nylon fibers  
17 per well, equivalent to 122  $\mu\text{g/ml}$  polyester and 39  $\mu\text{g/ml}$  nylon, as this concentration had  
18 clear effects and was on the lower end of the spectrum of concentrations used in other  
19 studies (7).

20 Murine lung organoids develop into two distinct phenotypes, i.e. acetylated  $\alpha$ -tubulin-  
21 positive airway organoids (Figure 2A) and prosurfactant protein C-positive alveolar  
22 organoids (Figure 2B). We assessed the effects of our reference fibers on these two  
23 structures separately. Exposure during 14 days to either polyester or nylon microfibers  
24 significantly inhibited growth of these organoids (Figure 2C) compared to untreated  
25 controls, reflected by fewer organoids being present (Figure 2D and E). The effect of nylon  
26 on airway organoids was most profound of the two types of plastic and the two types of  
27 structures. Moreover, both airway and alveolar organoids were significantly smaller in size  
28 following nylon microfiber exposure as compared to untreated controls (Figure 2F and G).



29  
 30 **Figure 2. Effects of microplastic fibers on growth of murine lung organoids.** Light  
 31 microscopy images and fluorescence photographs of (A) acetylated  $\alpha$ -tubulin-positive  
 32 airway organoids (red) and (B) prosurfactant protein C-positive alveolar organoids  
 33 (green). Nuclei were counterstained with DAPI (blue). (C) Representative light microscopy  
 34 images of the different treatment conditions. Yellow arrows in the light microscopy images  
 35 indicate airway organoids, whereas cyan arrows indicate alveolar organoids. (D and E)  
 36 Quantification of the numbers and (F and G) quantification of the sizes of airway and  
 37 alveolar lung organoids exposed for 14 days to no fibers, 5000 polyester, or 5000 nylon  
 38 fibers (equivalent to 122  $\mu\text{g}/\text{ml}$  polyester or 39  $\mu\text{g}/\text{ml}$  polyester,  $n=12$  independent  
 39 isolations). Groups were compared using a Friedman test with Dunn's correction for  
 40 multiple testing.  $P<0.05$  was considered significant.

41 Similar results were observed in human lung organoids, that mainly developed into alveolar  
42 prosurfactant protein C-positive organoids or mixed alveolar/airway organoids positive for  
43 both acetylated  $\alpha$ -tubulin and prosurfactant protein C (Figure 3A). 14 day-exposure to nylon  
44 microfibers resulted in significantly fewer human lung organoids (Figure 3B and C),  
45 whereas the effects of polyester on organoid growth were less profound. The size of the  
46 organoids was not affected by the presence of nylon microfibers (Figure 3D).



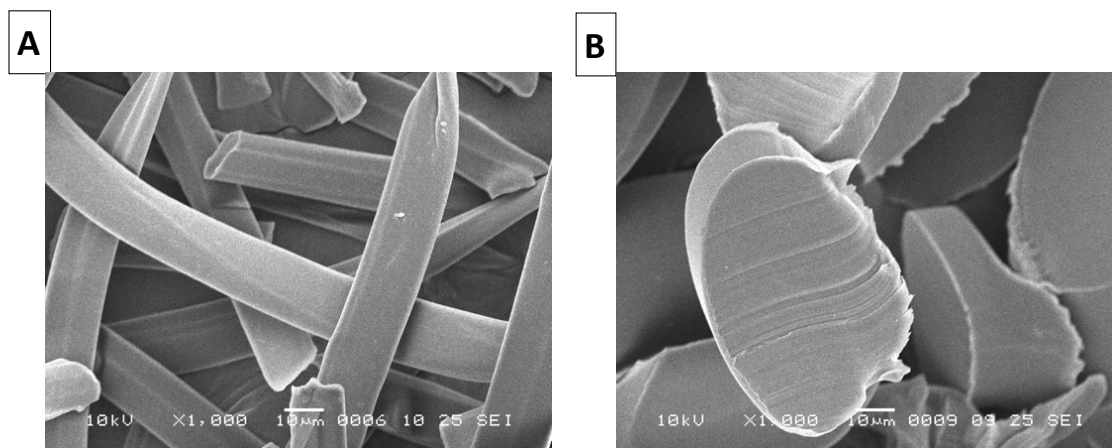
47 **Figure 3. Influence of microplastic fibers on growth of human lung organoids.** (A) The  
48 morphology of the alveolar prosurfactant protein C-positive organoids (green) and mixed  
49 acetylated  $\alpha$ -tubulin/prosurfactant protein C-positive organoids (orange) as shown by light  
50 and fluorescence microscopy. Nuclei were counterstained with DAPI (blue). (B)  
51 Representative light microscopy images of all treatment conditions. Cyan arrows indicate  
52 lung organoids. (C) Quantification of the numbers and (D) sizes of human lung organoids  
53 following 14-day exposure to either no microfibers, 5000 polyester, or 5000 nylon fibers  
54 (equivalent to 122  $\mu$ g/ml polyester or 39  $\mu$ g/ml polyester,  $n=7$  independent isolations).



55 *Groups were compared using a Friedman test with Dunn's correction for multiple testing.*  
56 *P<0.05 was considered significant.*

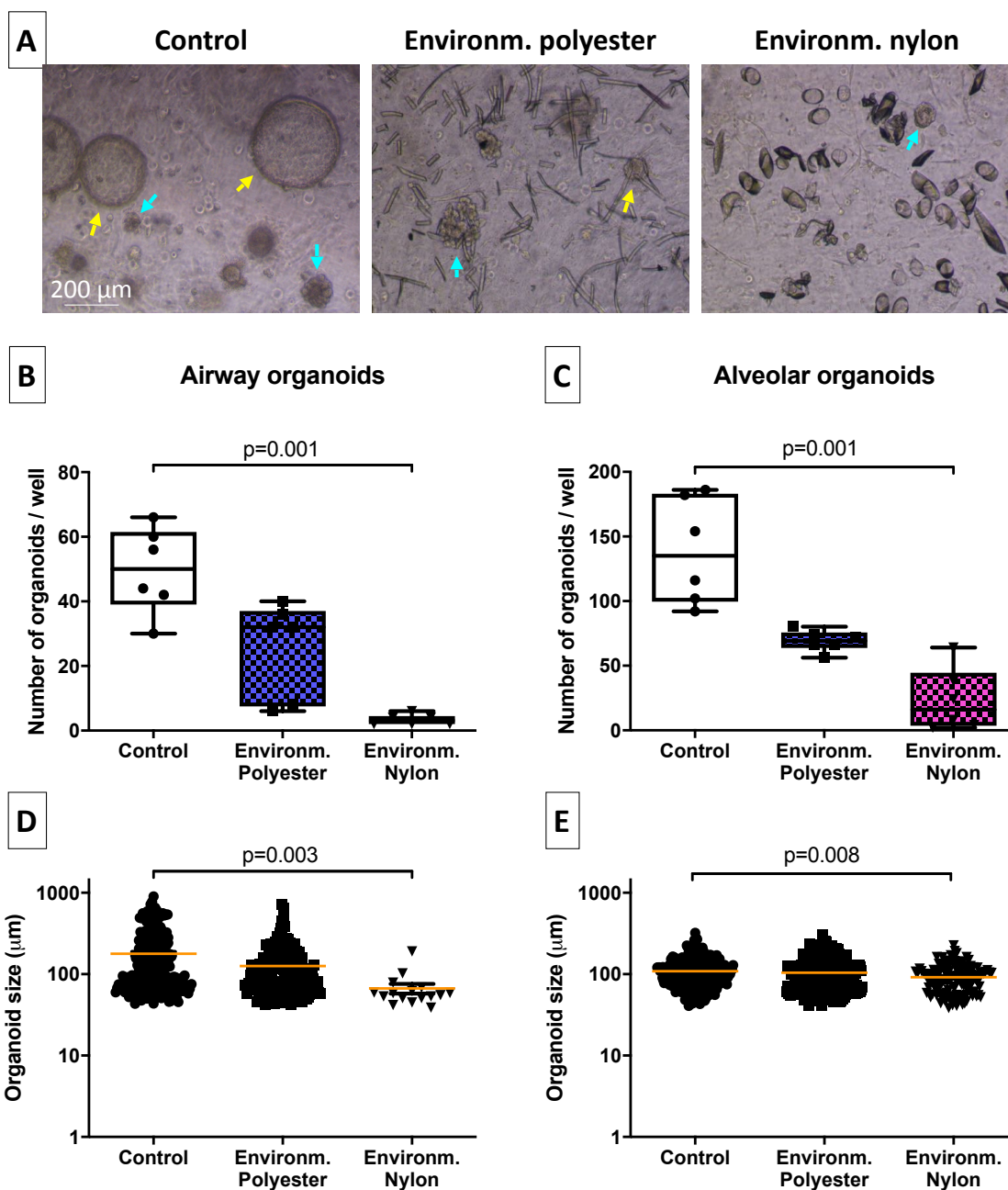
### 58 **Environmental microplastic fibers impaired lung organoid growth as well**

59 In addition to reference microfibers, we also assessed effects of environmentally relevant  
60 polyester and nylon fibers on murine lung organoids. These were made from white  
61 polyester and nylon fabrics purchased in a local fabric store and cut to sizes approximating  
62 the reference fibers. First, we characterized morphology and chemical composition of these  
63 fibers. For polyester, we observed a more heterogeneous size distribution as compared to  
64 the reference fibers, with fibers having a median size of 17x63  $\mu\text{m}$  (Figure 4A, Table S2),  
65 but a comparable EDX and  $\mu\text{FTIR}$  spectrum (Figure S3A and B). The nylon fibers had a  
66 disk-shaped appearance but similar dimensions as the reference nylon fibers (median 57x20  
67  $\mu\text{m}$ , Table S2). The EDX analysis revealed the expected C, N and O peaks for nylon (Figure  
68 S3C) and the  $\mu\text{FTIR}$  spectrum showed characteristic nylon absorbance peaks (Figure S3D).



69  
70 **Figure 4. Morphology of environmental microplastic fibers.** Representative SEM pictures  
71 of (A) polyester microfibers (17x63  $\mu\text{m}$ ) and (B) nylon microfibers (57x20  $\mu\text{m}$ ).

72  
73 When exposing murine lung organoids to these environmentally relevant textile fibers, we  
74 obtained results similar to the reference fibers: exposure to environmental nylon microfibers  
75 resulted in markedly fewer lung organoids (Figure 5A, B and C) as well as smaller  
76 organoids (Figure 5D and E).

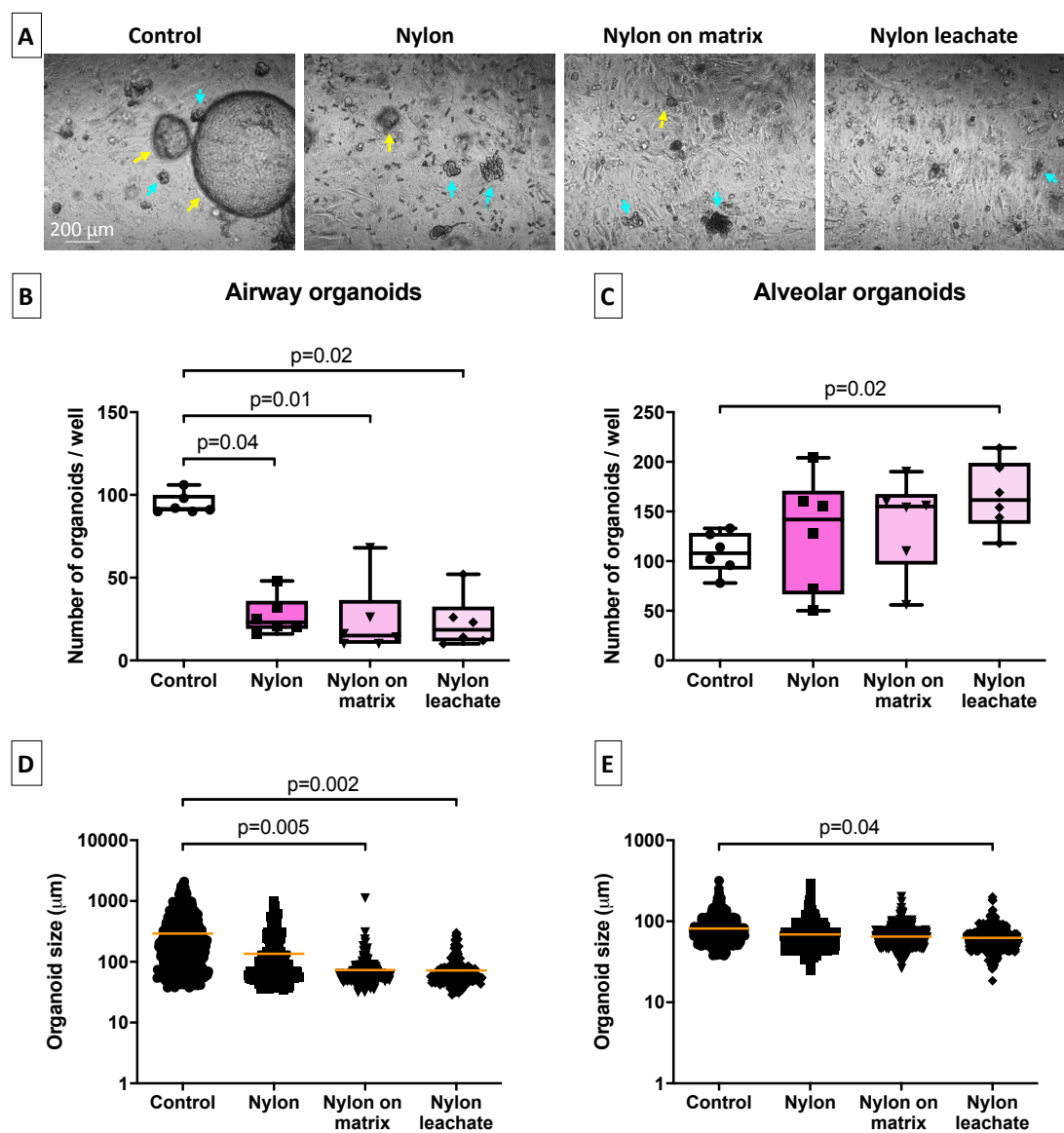


77  
78 **Figure 5: Effect of environmentally relevant textile fibers on growth of murine lung**  
79 **organoids.** (A) Representative light microscopy images of all treatment conditions. Yellow  
80 arrows in the light microscopy images indicate airway organoids, whereas cyan arrows  
81 indicate alveolar organoids. (B and C) Quantification of the numbers and (D and E) sizes  
82 of airway and alveolar organoids ( $n=6$  independent isolations) following 14-day exposure  
83 to either no microfibers, 5000 polyester or 5000 nylon microfibers (approximately  
84 equivalent to 189  $\mu\text{g}/\text{ml}$  polyester or 531  $\mu\text{g}/\text{ml}$  nylon). Groups were compared using a  
85 Friedman test with Dunn's correction for multiple testing.  $P<0.05$  was considered  
86 significant.

## 87 **Leaching nylon components caused a reduction in lung organoid growth**

88 Since organoid growth was mostly affected by both reference and environmentally relevant  
89 nylon microfibers, we investigated whether this inhibition was caused by the physical  
90 presence of fibers nearby the cells or by leaching components from these nylon fibers.

91 Therefore, we added nylon reference microfibers either on top of the 3D matrigel matrix  
92 after it had set, thereby preventing direct contact with the cells, or added leachate of these  
93 fibers to the medium surrounding the matrigel matrix. We found significantly fewer airway  
94 organoids in the presence of nylon microfibers on top of the gel or their leachate (Figure 6A  
95 and B), while the number of alveolar organoids was unaffected (fibers on top) or even  
96 induced (leachate, Figure 6A and C). Additionally, the size of these airway organoids was  
97 smaller as compared to untreated control organoids (Figure 6D), while only slightly  
98 inhibiting the size of the alveolar organoids (Figure 6E). These data suggest that specifically  
99 airway epithelial growth is inhibited by components leaching from nylon microplastics.

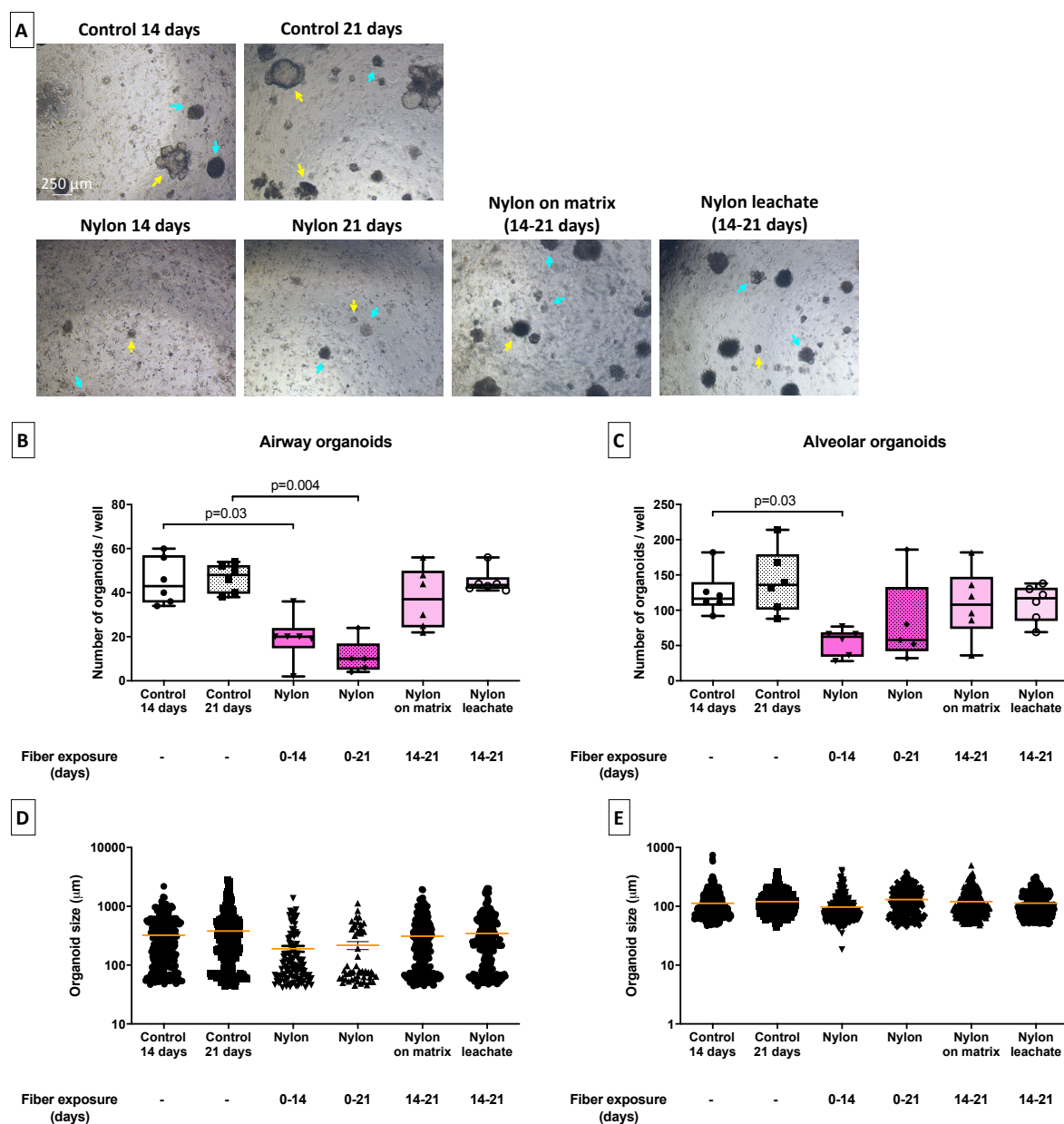


**Figure 6. Impact of nylon reference microfibers and their leaching components on growth of murine lung organoids.** (A) Representative light microscopy images of all treatment conditions. Yellow arrows in the light microscopy images indicate airway organoids, whereas cyan arrows indicate alveolar organoids. (B and C) Quantification of the numbers and (D and E) sizes of airway and alveolar organoids following either direct exposure to 5000 nylon microfibers or indirect exposure to nylon by adding 5000 microfibers (equivalent to 39  $\mu\text{g}/\text{ml}$  nylon) on top of the 3D matrigel matrix or by adding nylon leachate to the culture medium ( $n=6$  independent isolations). Groups were compared using a Friedman test with Dunn's correction for multiple testing.  $P<0.05$  was considered significant.

12 We next determined the chemical identity of the components leaching from nylon reference  
13 microfibers. Mass spectrometry analysis revealed high concentrations of cyclic nylon  
14 oligomers (mono-, di- and trimers) in the leachate of nylon microfibers (Figure S4A), which  
15 are known to develop as by-products during the production of nylon (36). Remarkably,  
16 when exposing murine lung organoids to different concentrations of these isolated  
17 oligomers separately or in combination, we observed no effects on either number or size of  
18 organoids (Figure S4B-E showing the highest concentration that has been tested). These  
19 data suggest that other components in nylon leachate are causing the inhibitory effects on  
20 organoid growth.

### 21 22 **Leaching nylon components mainly affected developing organoids**

23 As our experimental set-up specifically studied effects of microplastic fibers on developing  
24 organoids, we next studied whether already-developed, mature organoids were also affected  
25 by nylon microplastics. We therefore exposed organoids to nylon reference microfibers  
26 during organoid formation as before (14-day incubation) and we additionally exposed fully  
27 developed 14-day organoids to microfibers on top of the 3D matrigel matrix or to nylon  
28 leachate for an additional 7 days. Interestingly, our data showed that the compounds  
29 leaching from nylon mostly inhibited developing organoids and not already-developed  
30 organoids (Figure 7A), as reflected by unchanged numbers of organoids (Figure 7B and C)  
31 and unchanged sizes (Figure 7D and E) after treatment of developed organoids. This  
32 suggests that these nylon leachates are mostly harmful to differentiation of epithelial  
33 progenitors, but do not kill fully differentiated epithelial cells.



34  
35 **Figure 7. Effects of nylon reference fiber leachate on growth of already-developed lung**  
36 **organoids.** (A) Representative light microscopy images of all treatment conditions. Yellow  
37 arrows in the light microscopy images indicate airway organoids, whereas cyan arrows  
38 indicate alveolar organoids. (B and C) Quantification of the numbers and (D and E) sizes  
39 of airway and alveolar organoids following exposure to no or 5000 nylon microfibers  
40 (equivalent to 39 µg/ml nylon) for 14 or 21 days. A set of other organoids developed without  
41 treatment for 14 days and were exposed to nylon by adding 5000 microfibers (equivalent to  
42 39 µg/ml nylon) on top of the 3D matrix or by adding nylon leachate to the culture medium  
43 for another 7 days (n=6 independent isolations). Groups were compared using a Kruskal-  
44 Wallis test with Dunn's correction for multiple testing.  $P < 0.05$  was considered significant.  
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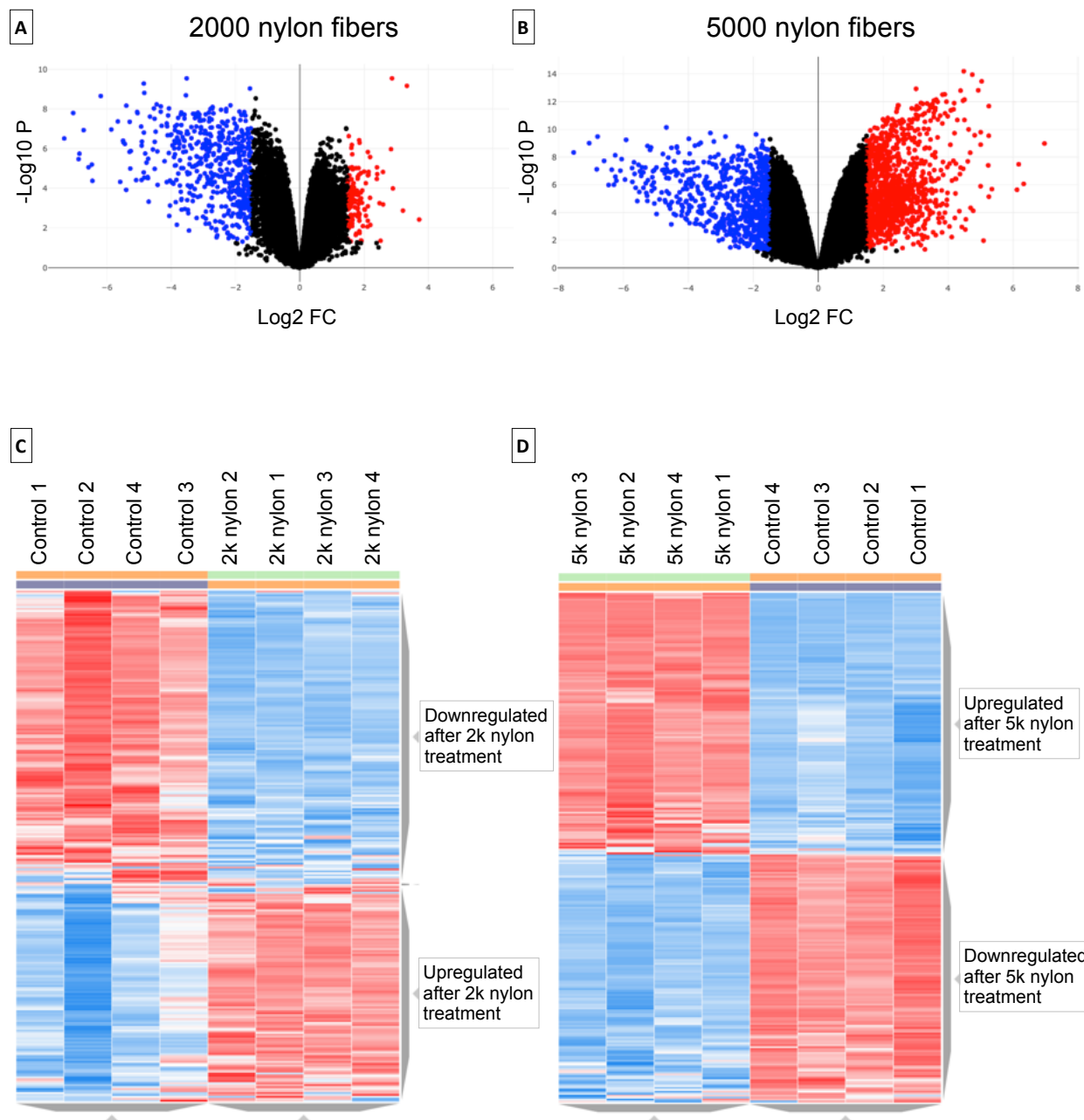
## 46 **Exposure to nylon inhibited epithelial development pathways and stimulated** 47 **expression of ribosome components**

48 To better understand the mechanisms behind the damaging effects of the leaching nylon  
49 components on in particular the growth of airway organoids, we performed bulk RNA-  
50 sequencing (RNAseq) analysis on EpCAM+ cells and fibroblasts resorted from organoid  
51 cultures exposed to two different concentrations of nylon fibers (2000 or 5000 fibers) or  
52 not. Both conditions had an enormous impact on epithelial gene expression as depicted by  
53 the volcano plots (Figure 8A-D). Exposure to 2000 nylon fibers (equivalent to 16 µg/ml  
54 nylon) resulted in 16455 transcripts being differentially expressed at least two-fold  
55 compared to nonexposed controls, with an q value <0.05 (p value corrected for the false  
56 discovery rate), with most being downregulated (Figure 8A and C). Exposure to 5000 nylon  
57 fibers (equivalent to 39 µg/ml nylon) resulted in 39395 transcripts being differentially  
58 expressed at least two-fold compared to nonexposed controls, with most being upregulated  
59 (Figure 8B and D).

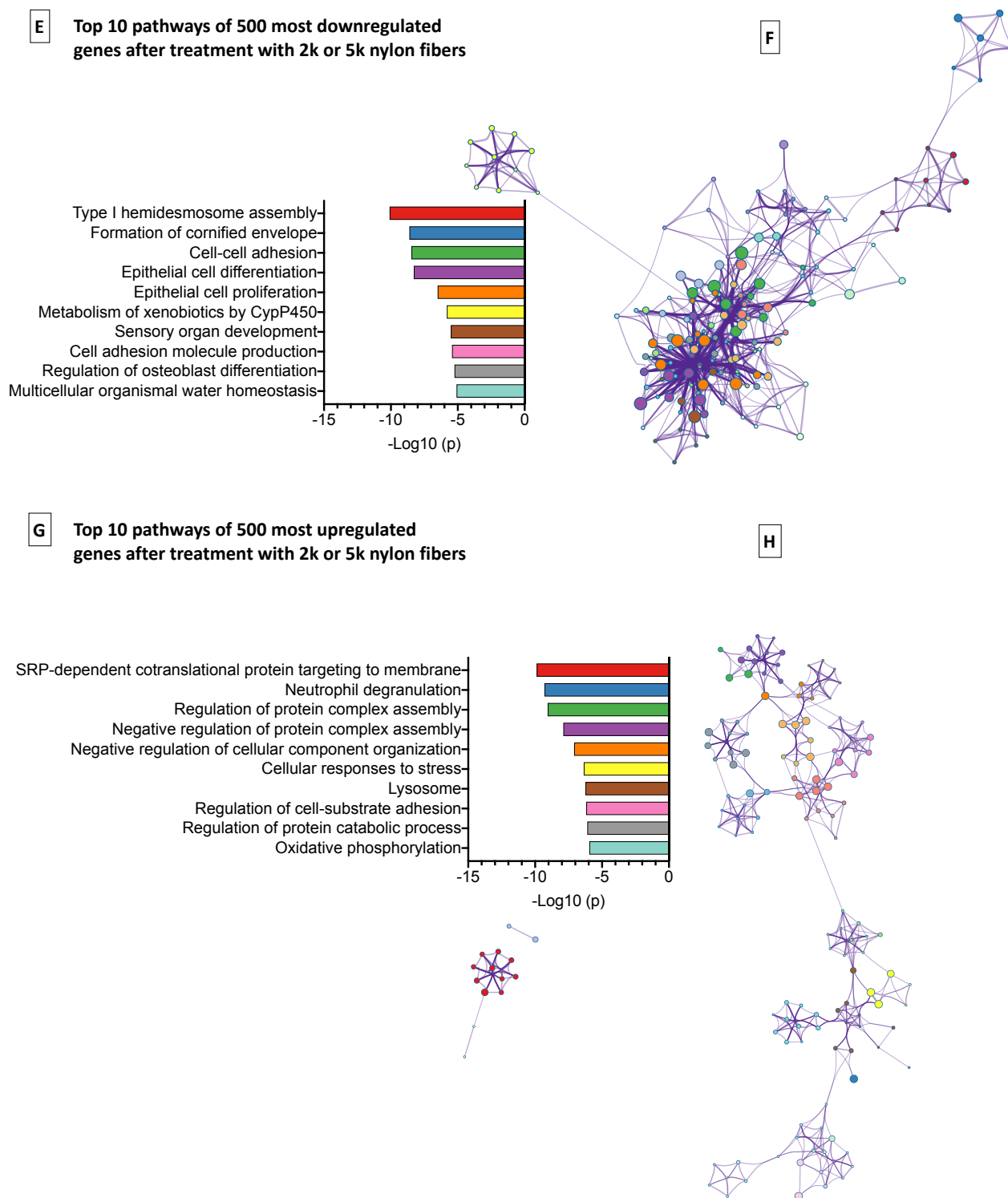
60 To reduce the number of transcripts, we then selected only those transcripts that had an  
61 average basemean expression of at least 10 and were significantly (q value <0.05) up or  
62 downregulated in both exposure conditions of 2000 and 5000 nylon fibers compared to  
63 nonexposed controls. This resulted in 10764 transcripts that were differentially expressed  
64 compared to nonexposed controls, with 5522 being downregulated and 5242 being  
65 upregulated. The downregulated transcripts were then sorted on the lowest q value for  
66 exposure to 2000 fibers and the upregulated transcripts were sorted on the lowest q value  
67 for exposure to 5000 fibers and the top 500 genes of each were used for pathway analysis  
68 using Metascape (37).

69 The top pathways identified for downregulated genes following nylon exposure were highly  
70 enriched for epithelial development and function (figure 8E-F), while the top pathways  
71 identified for upregulated genes were highly enriched for mRNA translation and protein  
72 synthesis (figure 8G-H).

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76  
 77 **Figure 8: RNAseq analysis of epithelial cells exposed to nylon or not. (A)** Volcano plot of  
 78 differentially expressed genes by epithelial cells exposed to 2000 nylon fibers (equivalent to  
 79 16  $\mu\text{g/ml}$  nylon) or not. **(B)** Volcano plot of differentially expressed genes by epithelial cells  
 80 exposed to 5000 nylon fibers (equivalent to 39  $\mu\text{g/ml}$  nylon) or not. Upregulated genes are  
 81 marked in red, downregulated genes in blue. Genes were selected with thresholds of fold  
 82 change  $>2$  and  $q < 0.05$ . **(C)** Unsupervised clustering heat map of epithelial cells exposed to

83 2000 nylon fibers (equivalent to 16  $\mu\text{g/ml}$  nylon) or not. **(D)** Unsupervised clustering heat  
84 map of epithelial cells exposed to 5000 nylon fibers (equivalent to 39  $\mu\text{g/ml}$  nylon) or not.  
85 **(E)** Metascape bar graphs of top 10 nonredundant enrichment clusters of genes  
86 downregulated by exposure to nylon ordered based on statistical significance ( $p$  value). **(F)**  
87 Metascape enrichment network visualization showing the intra-cluster and inter-cluster  
88 similarities of enriched terms of genes downregulated by exposure to nylon, up to ten terms  
89 per cluster. Cluster annotations colors are shown in bar graph of panel E. **(G)** Metascape  
90 bar graphs of top 10 nonredundant enrichment clusters of genes upregulated by exposure to  
91 nylon ordered based on statistical significance ( $p$  value). **(H)** Metascape enrichment  
92 network visualization showing the intra-cluster and inter-cluster similarities of enriched  
93 terms of genes upregulated by exposure to nylon, up to ten terms per cluster. Cluster  
94 annotations colors are shown in bar graph of panel E. 2k: 2000 fibers; 5k: 5000 fibers.

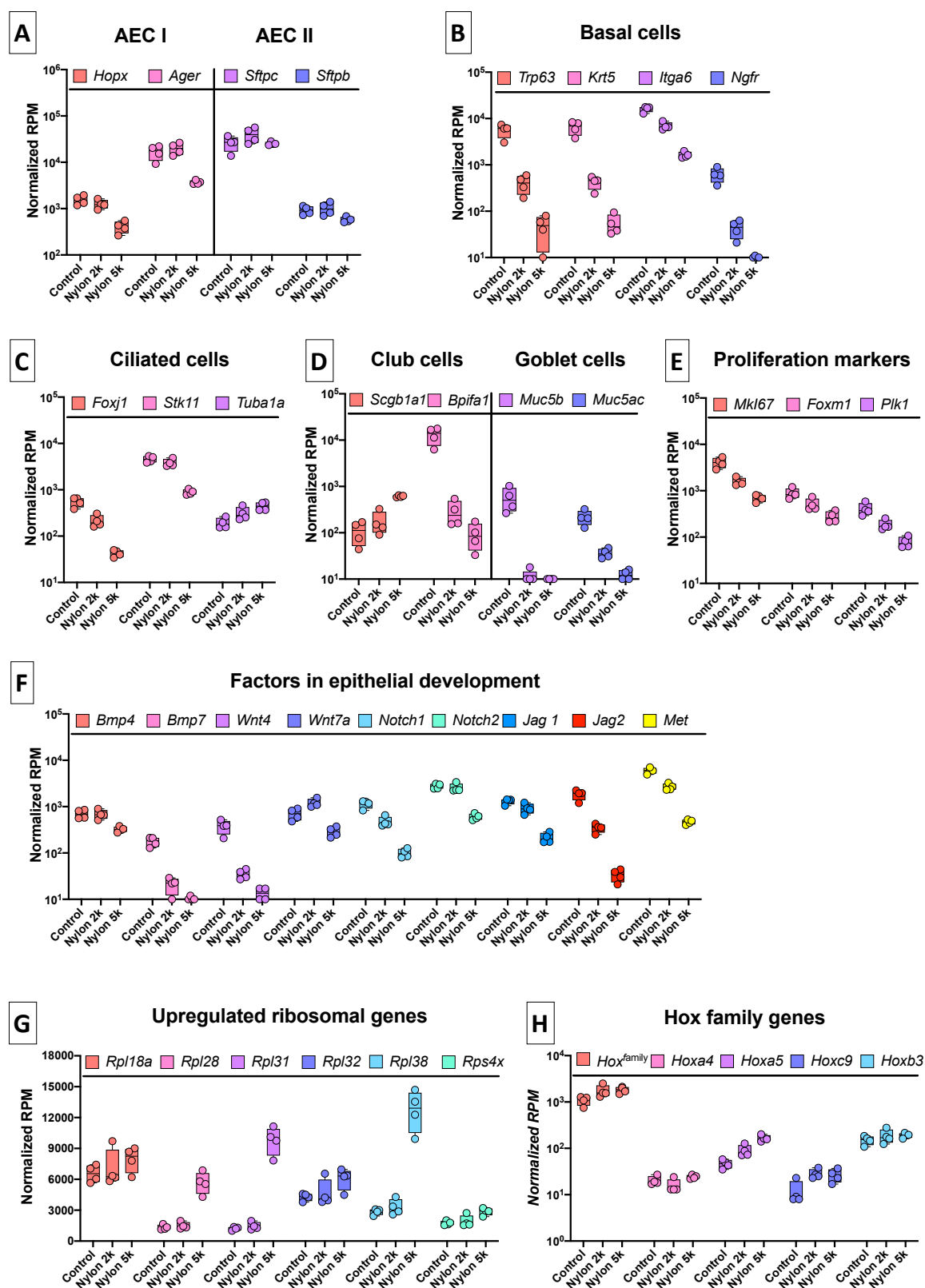
95  
96 We then investigated expression of individual genes in the top 5 enriched pathways for up  
97 and downregulated genes in more detail (Figures 8E-H). The top 5 enriched pathways for  
98 downregulated genes were type I hemidesmosome assembly, formation of cornified  
99 envelope, cell-cell adhesion, epithelial cell differentiation, and epithelial cell proliferation,  
00 while the top 5 pathways for upregulated genes were SRP-dependent cotranslational protein  
01 targeting to membrane, neutrophil degranulation, regulation of protein complex assembly,  
02 negative regulation of protein complex assembly, and negative regulation of cellular  
03 component organization.

04 We first investigated the downregulated genes and many of them represent important  
05 epithelial populations in the lung. We therefore investigated genes associated with specific  
06 epithelial populations, i.e. homeodomain-only protein homeobox (*Hopx*) and advanced  
07 glycosylation end-product specific receptor (*Ager*) for alveolar epithelial cell type I cells  
08 (AECI) and surfactant protein C (*Sftpc*) and surfactant protein B (*Sftpb*) for alveolar  
09 epithelial cell type II cells (AECII). For airway epithelial cell types we used transformation-  
10 related protein 63 (*Trp63*), keratin 5 (*Krt5*), integrin alpha-6 (*Itga6*), and nerve growth  
11 factor receptor (*Ngfr*) for basal cells, forkhead box J1 (*Foxj1*), serine/threonine kinase 11  
12 (*Stk11*) and, acetylated alpha tubulin (*Tuba1a*) for ciliated cells, secretoglobin family 1A  
13 member 1 (*Scgb1a1*) and BPI fold containing family A member 1 (*Bpifal*) for club cells,  
14 and mucin 5 subtype B and subtype AC (*Muc5b*, *Muc5ac*) for goblet cells. The expression  
15 of these genes correlated well with our organoid findings that airway epithelial cell growth

16 was most affected by exposure to nylon fibers, while alveolar epithelial cell growth was less  
17 affected (Figure 9). Both AECI and AEC II genes (Figure 9A) were only marginally lower  
18 expressed after exposure to nylon while most genes for basal cells (Figure 9B), ciliated cells  
19 (Figure 9C), club cells and goblet cells (Figure 9D) were expressed at significantly lower  
20 levels compared to controls with two noticeable exceptions: ciliated cell marker acetylated  
21 alpha tubulin *Tuba1a* and club cell marker secretoglobin family 1A member 1 *Scgb1a1* that  
22 were expressed at significantly higher levels compared to nonexposed controls.

23 Proliferation markers like proliferation marker protein 67 (*Mki67*), forkhead box protein M1  
24 (*Foxm1*), and polo-like kinase 1 (*Plk1*, Figure 9E) confirmed this general lack of  
25 proliferation in epithelial cells as all three were expressed at lower levels in a dose-  
26 dependent manner after exposure to nylon fibers. Other factors regulating epithelial  
27 development (38-45) from the top 5 pathways are depicted in figure 9F and all show the  
28 same dose-dependent lower expression after exposure to nylon as the proliferation markers  
29 with the exception of bone morphogenetic protein 4 (*Bmp4*), wingless-type MMTV  
30 integration site family, member 7a (*Wnt7a*), and neurogenic locus notch homolog protein 2  
31 (*Notch2*) that were only expressed at lower levels after exposure to 5000 nylon fibers  
32 compared to nonexposed controls.

33 To exclude the possibility that these effects on epithelial cells were the result of a decreased  
34 support function from fibroblasts, for instance by nylon selectively killing fibroblasts or  
35 inhibiting the expression of important growth factors, we separately analyzed the resorted  
36 fibroblast fraction for expression of proliferation genes and important growth factors, i.e.  
37 *Mki67*, *Foxm1*, *Plk1*, fibroblast growth factors 2, 7, and 10 (*Fgf2*, *Fgf7*, and *Fgf10*), *Wnt2*,  
38 *Wnt5a*, and hepatocyte growth factor (*Hgf*) (33). None of these genes were negatively  
39 affected in fibroblasts after exposure to nylon fibers compared to untreated controls and  
40 expression of most actually went up slightly (Figure S5).



41  
42 **Figure 9: Expression profiles of individual genes from the pathway analyses. (A) Genes**  
43 **highly expressed by alveolar epithelial cells type I (AECI) and type II (AECII). (B) Genes**  
44 **highly expressed in basal cells. (C) Genes highly expressed in ciliated cells. (D) Genes**  
45 **highly expressed in club cells and goblet cells. (E) Genes associated with proliferation. (F)**

46 *Genes encoding factors important for epithelial development. (G) Genes encoding*  
47 *ribosomal proteins. (H) Genes encoding Hox family genes.*

48  
49 The genes most prominently upregulated after exposure to nylon fibers were mostly  
50 encoding for ribosomal proteins, with ribosomal protein L28 (*Rpl28*), L31 (*Rpl31*), and L38  
51 (*Rpl38*) being most profoundly upregulated (Figure 9G). Ribosomal proteins are a large  
52 family of proteins and essential parts of ribosomes translating mRNA to protein. Recent  
53 data has shown that heterogeneity in ribosomal protein composition within ribosomes  
54 makes them selective for translating subpools of transcripts (46). For instance, Rpl38 has  
55 been shown to regulate translation of the homeobox (*Hox*) genes, that are key in anatomical  
56 development (47). We therefore also investigated the expression of all *Hox* genes and some  
57 specific members of this family involved in lung epithelial development in more detail  
58 (Figure 9H) (48, 49). After exposure to nylon fibers, expression of Hox family members  
59 was significantly higher and this pattern was seen for highlighted members *Hoxa4*, *Hoxa5*,  
60 *Hoxc9*, and *Hoxb3* as well.

## 62 Discussion

63 Recent reports have shown that man-made fibers are ubiquitously present in indoor air (9,  
64 50-53). It is estimated that approximately 30% of those indoor fibers are of plastic origin,  
65 particularly from textiles (9, 50-53). The lungs are continuously exposed to this airborne  
66 microplastic pollution (54), but the consequences of common household exposure on our  
67 lungs are unclear. High occupational exposure to nylon microfibers has been linked to the  
68 development of airway and interstitial lung disease (15-23), suggesting that as microplastic  
69 pollution accumulates, establishing the effects of inhaling this pollution becomes  
70 increasingly important. In our present work, we found that both polyester and nylon  
71 microfibers negatively affected the growth and development of human and murine lung  
72 organoids, with nylon being the most harmful. Already established lung organoids were not  
73 affected and therefore our results may be of particular importance for young children with  
74 developing airways and for people undergoing high levels of epithelial repair, such as  
75 people with respiratory diseases.

76  
77 We particularly focused on polyester and nylon, because these are the most abundant types  
78 of microplastics indoors (9, 50-53). As we spend the majority of our time indoors, we may

79 therefore be exposed most to these types of microplastics (55). Nylon was found to be the  
80 most consistently harmful for growth of airway organoids and was less inhibitory for  
81 growth of alveolar organoids, while polyester affected both types equally but less  
82 profoundly than nylon. This was the case for our reference fibers as well as environmentally  
83 relevant fibers made from fabrics purchased in a local fabric store. The lung organoids we  
84 used in these studies self-assemble from lung epithelial progenitor/stem cells isolated from  
85 adult lung tissue. The main populations of epithelial progenitor/stem cells in these lung  
86 tissues are basal cells and club cells in the airways and AECII in alveoli. The basal cell  
87 progenitors can give rise to all important epithelial cells lining the airways, among which  
88 ciliated cells, goblet cells, and club cells. Club cells can develop into ciliated cells, goblet  
89 cells, and AECII (56, 57). The growth of organoids from these progenitor cells is supported  
90 by proliferation and differentiation enhancers produced by epithelial cells themselves and  
91 fibroblasts also present in our 3D cultures (58).

92 The gene expression pathway analysis after nylon exposure confirmed inhibition of  
93 important pathways for epithelial regeneration. Expression of basal cell marker genes were  
94 impressively and dose-dependently downregulated by nylon exposure, as were genes for  
95 signaling molecules essential for epithelial growth and development, like *Notch1* and  
96 *Notch2* and their ligands Jagged 1 (*Jag1*) and 2 (*Jag2*) (43-45), *Bmp4* and *Bmp7* (40-42),  
97 *Wnt4* and *Wnt7a* (59, 60), and the receptor for hepatocyte growth factor, *Met*. The lower  
98 expression of basal cell-specific markers and essential factors that are needed for  
99 differentiation of other cell types like ciliated and goblet cells may explain why the growth  
00 of in particular airway organoids was inhibited most by nylon.

01 The gene expression analysis also confirmed that growth of alveolar organoids was affected  
02 less by nylon exposure and even appeared induced after treatment with leachate or lower  
03 numbers of nylon fibers. Genetic markers of both AECI and II were either stable or only  
04 slightly affected by the highest concentration of nylon. The explanation for this finding may  
05 be found in the downregulation of Notch signaling pathway members by nylon fibers.  
06 Multiple studies have shown that Notch signaling is required for development of airway  
07 epithelial cells, most specifically goblet cells, whereas disruption of this signaling boost  
08 alveolar epithelial development (61, 62). Both *Notch1* and *Notch2*, as well as their ligands  
09 *Jag1* and *Jag2* were expressed at significantly lower levels after nylon treatment, suggesting  
10 disruption of Notch signaling may be responsible for the divergent effect nylon has on  
11 airway versus alveolar epithelial growth.

12 The Notch pathway, incidentally, is also important for the development of club cells (63).  
13 Morimoto and colleagues showed that *Notch2* was involved in the decision between club  
14 cell or ciliated cell development. Interestingly, the gene expression data for club cells and  
15 ciliated cells were somewhat ambiguous, with important markers for these cell types having  
16 higher expression (*Scgbl1a1* for club cells and *Tub1a1* for ciliated cells) and others having  
17 lower expression (*Bpifal* for club cells and *Foxj1* and *Stk11* for ciliated cells) after nylon  
18 exposure. This may suggest that the lower expression of *Notch2* may be hampering the cell  
19 fate decision between club and ciliated cells in some way, resulting in improperly  
20 differentiated cell types. In combination with the inhibited development of basal epithelial  
21 cells and goblet cells, this altered airway epithelial differentiation may explain the  
22 bronchiolitis found in nylon flock workers and rats exposed to nylon (64-66).

23  
24 For nylon, the negative effect we found on development of particularly airway organoids  
25 was caused by components leaching from these fibers. As the most abundant components in  
26 this leachate, the cyclic oligomers, were not responsible for this effect, we used RNAseq  
27 analysis to look for signatures of other components. Recent work by Sait and Sørensen and  
28 colleagues showed that the most abundant chemicals leaching from nylon are bisphenol A  
29 and benzophenone-3 (67, 68). Experiments incubating organoids with benzophenone-3  
30 showed some effect on organoid growth but mostly on alveolar development suggesting this  
31 was not the major culprit in our leachate (data not shown). Interestingly, however, a study  
32 investigating effects of bisphenol A in fruit flies found that exposure of these flies to  
33 bisphenol A specifically upregulated ribosome-associated genes (69), similar to what we  
34 found in our study. The association of these *Rpl* genes with the *Hox* family genes is of  
35 particular interest for lung epithelial development. *Rpl38* was one of the three most  
36 upregulated *Rpl* genes by nylon and was shown to interact with a specific subset of *Hox*  
37 genes including *Hoxa4*, *Hoxa5*, and *Hoxb3* (47). These have all been associated with  
38 epithelial differentiation, with *Hoxa5* taking a center stage in goblet versus club cell  
39 differentiation (48, 49). Boucherat and colleagues showed that loss of *Hoxa5* drove  
40 epithelial differentiation towards goblet cell differentiation at the expense of club cell  
41 differentiation and *Scgbl1a1* expression (48). Remarkably, *Hoxa5* was expressed at  
42 significantly higher levels in our dataset in a dose-dependent manner after exposure to  
43 nylon and we also found a matching dose-dependent increase in *Scgbl1a1* and decrease in  
44 *Muc5ac* and *Muc5b* expression. Of note, bisphenol A exposure was also found to inhibit

45 expression of Notch pathway members in rat brain, suggesting bisphenol A could also  
46 contribute to the aberrant epithelial cell differentiation through the Notch pathway (70).  
47 Taken together our data suggest that component (s) in nylon leachate, maybe bisphenols,  
48 may be skewing differentiation of epithelial progenitors away from airway epithelial cells  
49 possibly through *Hoxa5* and/or *Notch*. However, work from van Winkle and colleagues  
50 showed that fetal exposure of rhesus macaques to bisphenol A resulted in higher expression  
51 of both *Scgb1a1* and *Muc5b/Muc5ac* (71) in lung tissue, suggesting bisphenol A exposure is  
52 not the only explanation for the effects we found and further investigating into components  
53 of the nylon leachate are warranted.

54  
55 By using lung organoids we were able to test many different conditions and factors related  
56 to microplastic exposure on epithelial development and repair mechanisms. In addition, this  
57 also gave us the opportunity to directly study human lung epithelial repair, showing us these  
58 effects of microplastic textile fibers are relevant for human epithelial differentiation and  
59 growth too. Despite these strengths (32, 33, 58, 72, 73), lung organoids are a relatively  
60 simple model of lung tissue and lack the immune and endothelial compartment present *in*  
61 *vivo*. Especially having the immune compartment present could alter how lung tissue  
62 responds to these microplastic fibers. For example, innate immune cells like macrophages  
63 are also one of the first cells to come into contact with microplastic fibers following  
64 inhalation and macrophages are known to respond strongly to inhaled particles and fibers  
65 (74) and are also important for lung repair (75). It is therefore recommended to include lung  
66 macrophages in lung organoid cultures in future studies as was done before by Choi *et al.*<sup>53</sup>.  
67 This way, a more comprehensive view on the interaction between pivotal lung cells and  
68 microplastics can be obtained.

69  
70 The implication of our results for the human population is of high relevance. It is important  
71 to note that similar to the high occupational exposure in industry workers, the microplastic  
72 fiber doses as used in our *in vitro* experiments are most likely much higher than daily  
73 exposure for most people. Previous studies estimated that a male person with light activity  
74 may inhale around 272 microplastic particles per day based on air sampling using a  
75 breathing thermal manikin (54). It will be crucial to study in more detail how many and  
76 what kind of fibers deposit in which regions of the lungs and what fraction can still be  
77 cleared. The fibers we used in our study were not of a size that will penetrate deep into the



78 lungs. Generally only fibers with a diameter smaller than 3  $\mu\text{m}$  have this ability (35). Our  
79 fiber sizes were limited by the availability of polyester and nylon filaments of standardized  
80 small diameters. However, the sizes we did use can enter the lung and get trapped onto the  
81 airways, incidentally the place we found they appear to do most harm. It is therefore key  
82 that hazard characteristics of these textile microfibers are studied in more detail, in addition  
83 to exposure levels in indoor environments. A limitation here is the detection of  
84 microplastics, as smaller particles might escape current detection methods (10, 76).  
85 Especially our finding that epithelial differentiation and repair mechanisms are affected  
86 most by microplastics exposure, suggests airborne microplastics may be most harmful to  
87 young children with developing airways and to people undergoing high levels of epithelial  
88 repair. These could be people with a chronic lung disease or even healthy individuals  
89 suffering from a seasonal respiratory virus infection.

90  
91 In conclusion, with the ongoing and growing use of plastics, potential associated health  
92 problems in the human population may also increase. The results of the present study  
93 strongly encourage to look in more detail at both the hazard and the exposure, and outcomes  
94 of these experiments will be valuable to advise organizations such as the World Health  
95 Organization and Science Advice for Policy by European Academies who have recently  
96 stated that more research is urgently needed<sup>21,22</sup>. Importantly, future research should focus  
97 on examining the presence and number of such fibers both in our indoor environment and in  
98 human lung tissue, to better estimate the actual risk of these fibers to human health.

## 02 **Materials and Methods**

03

### 04 **Production of microfibers and leachate**

#### 05 *Reference microfibers and leachate*

06 Microfibers of standardized dimensions were produced as described before (34). In short,  
07 polyester and nylon fibers (both Goodfellow, UK) with filament diameters of  $14\pm 3.5\ \mu\text{m}$   
08 and  $10\pm 2.5\ \mu\text{m}$  respectively were aligned by wrapping them around a custom-made spool,  
09 coated with a thin layer of cryo compound (KP-CryoCompound, VWR International B.V.,  
10 PA, USA) and frozen. Aligned fibers were cut into similar length parts ( $\sim 2\ \text{cm}$ ) using a  
11 scalpel (Swann-Morton, UK) and moulded onto a compact block that was oriented  
12 perpendicular to the base of a cryomicrotome (Microm HM 525, Thermo Fisher Scientific,  
13 MA, USA). Microfibers were cut at lengths of  $50\ \mu\text{m}$  for polyester and  $30\ \mu\text{m}$  for nylon,  
14 after which the fibers were thawed, washed with water through a  $120\ \mu\text{m}$  filter (Merck  
15 Millipore, MA, USA) to remove miscut fibers and contaminants, collected by vacuum  
16 filtration using  $8\ \mu\text{m}$  polycarbonate membrane filters (Sterlitech, WA, USA) and stored dry  
17 at  $-20^\circ\text{C}$ .

18 Nylon leachate was produced by incubation of nylon reference microfibers in phosphate  
19 buffered saline (PBS) for 7 days at  $37^\circ\text{C}$  in the dark, followed by filtration using a  $0.2\ \mu\text{m}$   
20 syringe filter (GE Healthcare Life Sciences, UK). The leachate was stored at  $-20^\circ\text{C}$  until  
21 further use.

#### 22 *Environmental microfibers*

23 Environmental polyester and nylon textile microfibers were prepared from commercially  
24 available pure fabrics. White polyester fabric was washed at  $40^\circ\text{C}$  in a washing machine  
25 (Samsung, South Korea) and dried in a tumble dryer (Whirlpool, MI, US). Fibers with an  
26 estimated filament diameter of  $15\ \mu\text{m}$  were collected on the filter of the tumble dryer and  
27 subsequently frozen with cryo compound and sectioned into lengths of  $50\ \mu\text{m}$  using a  
28 cryomicrotome. White nylon fabric (estimated filament diameter of  $40\ \mu\text{m}$ ) was cut into  
29 small squares, stacked, frozen with cryo compound, and cut into lengths of  $12\ \mu\text{m}$ . All  
30 microfibers were thawed, washed with water through a  $120\ \mu\text{m}$  filter, collected by vacuum  
31 filtration ( $8\ \mu\text{m}$  filter) and finally stored at  $-20^\circ\text{C}$ .

32

## 33 **Characterization of microfibers and leachate**

### 34 *Scanning electron microscopy*

35 Samples were prepared for scanning electron microscopy (SEM) analysis on an aluminium  
36 sample holder using adhesive carbon-coated tape. Excessive microfibers were removed  
37 using pressurized air, after which the samples were sputter-coated with 10 nm of gold.  
38 Images were obtained using a JSM-6460 microscope (Jeol, Japan) at an acceleration voltage  
39 of 10 kV.

### 40 *Dimensions*

41 Digital photomicrographs were captured at 200× magnification using a Nikon Eclipse  
42 TS100 inverted microscope coupled to a Nikon Digital Sight DS-U3 microscope camera  
43 controller (both Japan), after which microfiber diameters and lengths (median of 200) were  
44 determined using NIS-Elements AR 4.00.03 software.

## 46 **Ethics**

### 47 *Animal experiments*

48 The experimental protocol for the use of mice for epithelial cell isolations was approved by  
49 the Animal Ethical Committee of the University of Groningen (The Netherlands), and all  
50 experiments were performed according to strict governmental and international guidelines  
51 on animal experimentation. C57BL/6 mice (8-14 weeks) were bred at the Central Animal  
52 Facility of the University Medical Center Groningen (UMCG) (IVD 15303-01-004).  
53 Animals received ad libitum normal diet with a 12 h light/dark cycle.

### 54 *Human lung tissue*

55 Histologically normal lung tissue was anonymously donated by individuals with COPD  
56 (n=6) or without COPD (n=1) undergoing surgery for lung cancer and not objecting to the  
57 use of their tissue. COPD patients included ex- and current- smoking individuals with  
58 GOLD stage I-IV disease (GOLD I=1, GOLD II=2, GOLD IV=3). Subjects with other lung  
59 diseases such as asthma, cystic fibrosis, or interstitial lung diseases were excluded. The  
60 study protocol was consistent with the Research Code of the University Medical Center  
61 Groningen (UMCG) and Dutch national ethical and professional guidelines  
62 ([www.federa.org](http://www.federa.org)). Sections of lung tissue of each patient were stained with a standard  
63 haematoxylin and eosin staining and checked for abnormalities by a lung pathologist.

## 64 **Cell cultures**

65 Mouse lung fibroblasts (CCL-206, ATCC, Wesel, Germany) or human lung fibroblasts  
66 (MRC5, ATCC, CCL-171) were cultured in 1:1 DMEM (Gibco, MD, USA) and Ham's F12  
67 (Lonza, Switzerland) or Ham's F12 respectively, both supplemented with 10% heat  
68 inactivated fetal bovine serum (FBS, GE Healthcare Life Sciences), 100 U/ml penicillin and  
69 100 µg/ml streptomycin, 2 mM L-glutamine and 2.5 µg/ml amphotericin B (all Gibco).  
70 Fibroblasts were cultured at 37°C under 5% CO<sub>2</sub> and humidified conditions. For use in  
71 organoid cultures, near-confluent cells were proliferation-inactivated by incubation with 10  
72 µg/ml mitomycin C (Sigma-Aldrich, MO, USA) in cell culture medium for 2 hours, after  
73 which they were washed in PBS, and left in normal medium for at least 1 hour before  
74 trypsinizing and counting.

## 75 **Lung epithelial cell isolation**

### 76 *Mouse lung epithelial cell isolation*

78 Epithelial cells were isolated from lungs of mice using antibody-coupled magnetic beads  
79 (microbeads) as described before (32, 33, 72). In short, mice were sacrificed under  
80 anaesthesia, after which the lungs were flushed with 0.9% NaCl and instilled with 75  
81 caseinolytic units/1.5 ml dispase (Corning, NY, USA). After 45 minutes of incubation at  
82 room temperature, the lobes (excluding trachea and extrapulmonary airways) were  
83 homogenized in DMEM containing 100 U/ml penicillin and 100 µg/ml streptomycin and 40  
84 µg/ml DNase1 (PanReac AppliChem, Germany), washed in DMEM (containing penicillin,  
85 streptomycin and DNase1), and the digested tissue was passed through a 100 µm cell  
86 strainer. The suspension was incubated for 20 minutes at 4°C with anti-CD31 and anti-  
87 CD45 microbeads in MACS buffer and magnetically sorted using LS columns. The  
88 CD31<sup>-</sup>/CD45<sup>-</sup> flow-through was incubated for 20 minutes at 4°C with anti-CD326  
89 (EpCAM) microbeads in MACS buffer, after which purified epithelial lung cells were  
90 obtained by positive selection using LS columns. CD326-positive epithelial cells were  
91 resuspended in CCL206 fibroblast medium, counted and seeded into matrigel immediately  
92 after isolation with equal numbers of CCL206 fibroblasts, as described below. All materials  
93 were purchased at Miltenyi Biotec (Germany) unless stated otherwise.

## 95 *Human lung epithelial cell isolation*

96 Human lung epithelial cells were isolated from lung tissue specimens obtained from  
97 patients. Peripheral lung tissue was minced and dissociated in DMEM-containing enzymes  
98 (Multi Tissue Dissociation Kit) at 37°C using a gentleMACS Octo Dissociator. The cell  
99 suspension was filtered (70 µm and 35 µm nylon strainer, respectively) prior to 20-minute  
00 incubation at 4°C with anti-CD31 and anti-CD45 microbeads in MACS buffer. The  
01 CD31<sup>-</sup>/CD45<sup>-</sup> fraction was obtained by negative selection using an AutoMACS. Epithelial  
02 cells were then isolated by positive selection after 20-minute incubation at 4°C with anti-  
03 CD326 (EpCAM) microbeads in MACS buffer. Human EpCAM<sup>+</sup> cells were resuspended  
04 in 1:1 DMEM and Ham's F12, supplemented with 10% heat inactivated FBS, 100 U/ml  
05 penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine and 2.5 µg/ml amphotericin B,  
06 counted and seeded into matrigel immediately after isolation with equal numbers of MRC5  
07 fibroblasts. All materials were purchased at Miltenyi Biotec unless stated otherwise.

## 08 09 **Lung organoid cultures**

10 Lung organoids were grown as previously described with minor modifications (32, 33, 72).  
11 For mouse lung organoids, 10,000 EpCAM<sup>+</sup> cells and 10,000 CCL206 fibroblasts were  
12 seeded, and for human lung organoids, 5,000 EpCAM<sup>+</sup> cells and 5,000 MRC5 fibroblasts  
13 were seeded in 100 µl growth factor-reduced matrigel matrix (Corning) diluted 1:1 in  
14 DMEM:Ham's F-12 1:1 containing 10% FBS, 100 U/ml penicillin and 100 µg/ml  
15 streptomycin, 2 mM L-glutamine and 2.5 µg/ml amphotericin B into transwell 24-well cell  
16 culture plate inserts (Corning). Organoids were cultured in DMEM:Ham's F-12 1:1  
17 supplemented with 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-  
18 glutamine, 2.5 µg/ml amphotericin B, 4 ml/l insulin-transferrin-selenium (Gibco), 25 µg/l  
19 recombinant mouse (Sigma-Aldrich) or human epithelial growth factor (EGF, Gibco) and  
20 300 µg/l bovine pituitary extract (Sigma-Aldrich). During the first 48h of culture, 10 µM Y-  
21 27632 (Axon Medchem, the Netherlands) was added to the medium.

22 A titration curve for polyester or nylon microfibers was made with 2000, 3000, 4000 or  
23 5000 fibers per well corresponding to approximately 49, 73, 98 and 122 µg/ml of polyester  
24 fibers or 16, 23, 31, and 39 µg/ml of nylon fibers. For all other experiments, 5000 polyester  
25 or nylon reference (equivalent to 122 µg/ml of polyester or 39 µg/ml of nylon) or  
26 environmental microfibers (equivalent to 122 µg/ml of polyester or 39 µg/ml of nylon and)

27 were used per condition. Fibers were in direct contact with developing organoids during 14  
28 days by mixing them with matrigel and cells prior to seeding in the insert, except for those  
29 experiments studying effects of leaching nylon components. In those cases, 5000 polyester  
30 and nylon reference fibers were added on top of the organoids, thereby excluding physical  
31 contact between the microfibers and the developing organoids, or equivalent amounts of  
32 fiber leachate were added to the medium during 14 days of organoid culture. For testing the  
33 effects of nylon oligomers, concentrations between 26.8 ng/ml and 53.6 µg/ml were used;  
34 the latter concentration being twice as high as the used fiber concentrations (5000 fibers per  
35 condition). Oligomers were synthesized and characterized as described in the  
36 supplementary materials and methods. All organoid cultures were maintained for 14 to 21  
37 days at 37 °C under 5% CO<sub>2</sub> and humidified conditions. Medium was refreshed 3 times per  
38 week.

39 Organoid colony forming efficiency was quantified by manually counting the total number  
40 of organoids per well after 14 or 21 days of culturing using a light microscope at 100×  
41 magnification. For mouse organoids, a distinction was made between airway and alveolar  
42 organoids, whereas for human organoids only one organoid phenotype was distinguished.  
43 The diameter of the organoids was measured using a light microscope (Nikon, Eclipse Ti),  
44 only including organoids larger than 50 µm in diameter, with a maximum of 50 organoids  
45 per phenotype per well.

### 46 47 **Immunofluorescence staining**

48 Organoid cultures in matrigel were washed with PBS, fixed in ice-cold 1:1 acetone and  
49 methanol (both Biosolve Chimie, France) for 15 minutes at -20°C and washed again with  
50 PBS after which aspecific antibody-binding was blocked for 2 hours in 5% bovine serum  
51 albumin (BSA, Sigma-Aldrich). Organoids were incubated overnight at 4°C with the  
52 primary antibodies (mouse anti-acetylated α-tubulin (Santa Cruz, TX, USA) and rabbit anti-  
53 prosurfactant protein C (Merck, Germany)) both diluted 1:200 in 0.1% BSA and 0.1%  
54 Triton (Sigma-Aldrich) in PBS. Next, after extensive but gentle washing with PBS,  
55 organoids were incubated with the appropriate Alexa-conjugated secondary antibody for 2  
56 hours at room temperature (Alexa Fluor 488 donkey anti rabbit IgG and Alexa Fluor 568  
57 donkey anti mouse IgG, both Thermo Fisher Scientific) diluted 1:200 in 0.1% BSA and  
58 0.1% Triton in PBS. Organoid cultures were washed with PBS, excised using a scalpel,  
59 mounted on glass slides (Knittel, Germany) using mounting medium containing DAPI

(Sigma-Aldrich) and covered with a cover glass (VWR). Digital photomicrographs were captured at 200× magnification using a DM4000b fluorescence microscope and LAS V4.3 software (both Leica, Germany).

### **Isolation of epithelial cells and fibroblasts from organoid cultures**

200,000 mouse EpCAM+ primary cells and 200,000 CCL206 fibroblasts (n=4 independent isolations) were seeded in 1 ml matrigel diluted 1:1 in DMEM containing 10% FBS in 6-well plates (Greiner Bio-One, The Netherlands). 12.000 or 30.000 nylon reference microfibers were mixed with matrigel and cells prior to seeding. Murine organoid culture medium was maintained on top and refreshed every two days. After 7 days, organoid cultures were digested with 50 caseinolytic units/ml dispase for 30 minutes at 37°C, transferred to 15 ml tubes, washed with MACS BSA stock solution and autoMACS rinsing solution (both Miltenyi), and digested further with trypsin (VWR) diluted 1:5 in PBS for 5 minutes at 37°C. The cell suspension was then incubated for 20 minutes at 4°C with anti-EpCAM microbeads in MACS buffer, after which the suspension was passed through LS columns. Both the EpCAM+ (epithelial cells) and EpCAM- (fibroblasts) cell fractions were used for RNA isolation and subsequent sequencing.

### **Library preparation and RNA sequencing**

Total RNA was isolated from EpCAM+ and EPCAM- cell fractions using a Maxwell® LEV simply RNA Cells/Tissue kit (Promega, WI, USA) according to manufacturer's instructions. RNA concentrations were determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). Total RNA (300 ng) was used for library preparation. Paired-end sequencing was performed using a NextSeq 500 machine (Illumina; mate 1 up to 74 cycles and mate 2 up to 9 cycles). Mate 1 contained the first STL (stochastic labeling) barcode, followed by the first bases of the sequenced fragments, and mate 2 only contained the second STL barcode. The generated data were subsequently demultiplexed using sample-specific barcodes and changed into fastq files using bcl2fastq (Illumina; version 1.8.4). The quality of the data was assessed using FastQC (77). The STL barcodes of the first mate were separated from the sequenced fragments using an in-house Perl script. Low quality bases and parts of adapter sequences were removed with Cutadapt (version 1.12; settings: q=15, O=5, e=0.1, m=36) (78). Sequenced poly A tails were

92 removed as well, by using a poly T sequence as adapter sequence (T (100); reverse  
93 complement after sequencing). Reads shorter than 36 bases were discarded. The trimmed  
94 fragment sequences were subsequently aligned to all known murine cDNA sequences using  
95 HISAT2 (version 2.1.0; settings: k=1000, --norc). The number of reported alignments, k,  
96 was given a high number in order to not miss any alignment results (some genes have up to  
97 62 transcripts). Reads were only mapped to the forward strand (directional sequencing).  
98 Fragment sequences that mapped to multiple genes were removed (unknown origin). When  
99 fragments mapped to multiple transcripts from the same gene all but one were given a non-  
00 primary alignment flag by HISAT2 (flag 256). These flags were removed (subtraction of  
01 256) by the same Perl script in order to be able to use the Bash-based shell script  
02 (dqRNASeq; see below) that is provided by Bioo Scientific (Perkin Elmer, MA, USA).  
03 Fragments that mapped to multiple transcripts from the same gene were considered unique  
04 and were counted for each of the transcripts. The number of unique fragments (or read  
05 pairs) was determined for each transcript using the script provided by Bioo Scientific  
06 (dqRNASeq; settings: s=8, q=0, m=1). Counts that were used for further analysis were  
07 based on a unique combination of start and stop positions and barcodes (USS + STL).  
08

### 09 **Data analyses and statistics**

10 All statistics were performed with GraphPad Prism 9.0. Nonparametric testing was used to  
11 compare groups in all experiments. For comparison of multiple-groups, a Kruskal wallis or  
12 Friedman test was used for nonpaired or paired nonparametric data respectively with  
13 Dunn's correction for multiple testing. Differences in organoid size between groups were  
14 tested by using the average size of the organoids per independent experiment. Data are  
15 presented as median  $\pm$  range and p-values  $<0.05$  were considered significant.  
16

17 For RNA sequencing data, the principal component analyses were performed in R using the  
18 R package DESeq2 (version 1.26.0) (79) in order to visualize the overall effect of  
19 experimental covariates as well as batch effects (function: plotPCA). Differential gene  
20 expression analyses (treated vs. nontreated) was performed with the same R package  
21 (default settings; Negative Binomial GLM fitting and Wald statistics; design= $\sim$ condition),  
22 following standard normalization procedures. Transcripts with differential expression  $>2$   
23 (nylon-treated versus nontreated fibroblasts or epithelial cells) and an adjusted p-value



24 below 0.05 were considered differentially expressed in that specific cell type. Volcano plots  
25 and clustering heat maps were made using BioJupies (80). Pathway analysis was done using  
26 Metascape (37).

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09  
10  
11

## 12 **Supplementary Materials and methods**

13

### 14 **Energy dispersive X-ray spectroscopy**

15 Samples were prepared for energy dispersive X-ray (EDX) spectroscopy analysis on an  
16 aluminium sample holder using adhesive carbon coated tape. Excessive microfibers were  
17 removed using pressurized air, after which the samples were sputter coated with 10 nm of  
18 carbon. The EDX measurements were performed with a Tescan MAIA III GMH field  
19 emission scanning electron microscope (Czech Republic) equipped with a Bruker X-Flash  
20 30 mm<sup>2</sup> silicon drift energy dispersive X-ray microanalysis detector (MA, USA).

21

### 22 **Micro-Fourier transform infrared spectroscopy**

23 Micro-Fourier transform infrared spectroscopy ( $\mu$ FTIR) measurements were performed  
24 using a Thermo Nicolet iN10 micro Fourier transform infrared microscope. Spectra were  
25 recorded in the wavelength range from 4000 to 675 cm<sup>-1</sup> using a spectral resolution of 8  
26 cm<sup>-1</sup>. For the transmission measurements of the polyester reference material and the  
27 polyester and nylon environmental fibers, a small amount of the microfibers was  
28 transferred onto a diamond micro compression cell where the samples were compressed.  
29 For the reflection measurements of the nylon reference material and the polyester and  
30 nylon environmental fibers, a small portion of microfibers was suspended in water. The  
31 suspension was subsequently filtered over a gold coated 0.8  $\mu$ m polycarbonate filter (TJ  
32 Environmental, The Netherlands). A subset of approximately 100 fibers was individually  
33 measured directly on the filter using the reflection mode of the  $\mu$ -FTIR equipment.

34

### 35 **Extraction of nylon oligomers (mono-, di- and trimer)**

36 A round bottom flask containing 25.1 g cryogenically milled nylon powder (PA66, Sigma-  
37 Aldrich) and 500 ml methanol (VWR) was equipped with a reflux condenser and the  
38 suspension was stirred overnight at 50°C. Next, the suspension was cooled to  
39 approximately 30°C and filtered over a cellulose filter (VWR) to remove the remaining  
40 powder. The solvent was removed *in vacuo* using a rotary evaporator (Büchi rotavapor R-  
41 215, Switzerland). A white solid was obtained (yield 220 mg), of which the composition  
42 was determined using liquid chromatography/mass spectrometry (LC/MS) analysis.



### 43 **Isolation of nylon oligomers (mono-, di- and trimer)**

44 A column for silica gel chromatography ( $\varnothing$  30 mm, VWR) was charged with silica gel 60  
45 (27 g, 0.063-0.200 mm, Merck) and dichloromethane (DCM, VWR) as eluent. The crude  
46 extract containing the mixture of oligomers (200 mg) was added on top of the silica gel  
47 column and oligomers were separated on the column using DCM:methanol as eluent  
48 (DCM:MeOH gradient: 100:0  $\rightarrow$  90:10  $\rightarrow$  80:20), which resulted in complete separation  
49 of the oligomers. The collected fractions were checked for the presence of product using  
50 LC/MS. The fractions containing pure oligomer were combined, filtered over a glass filter  
51 (VWR) and subsequently the solvent was removed *in vacuo*. The obtained solids were  
52 further dried *in vacuo*, after which the pure oligomers were obtained as white solids. The  
53 structure of the oligomers was confirmed by  $^1\text{H}$  NMR spectroscopy.

### 55 **Liquid chromatography/mass spectrometry**

#### 56 *Qualitative analysis of nylon leachate and oligomers*

57 Qualitative analysis of nylon oligomers was performed with an Agilent 1260 series high-  
58 performance liquid chromatographer (CA, USA) equipped with a 100 x 2 mm, 3  $\mu\text{m}$   
59 Gemini NX-C18 110 Å LC Column (Phenomenex, Utrecht, The Netherlands), coupled  
60 with an Agilent 6410 triple quadrupole LC/MS with electron spray ionization (ESI) in  
61 positive SCAN mode. In addition, the LC/MS analysis of the nylon leachate was  
62 performed with the Agilent 1260 liquid chromatographer coupled to an Agilent 6460 triple  
63 quadrupole LC/MS with Jetstream ESI in positive SCAN mode. A sample volume of 5  $\mu\text{l}$   
64 was injected with a column temperature of 60  $^{\circ}\text{C}$  and a flow rate of 200  $\mu\text{l min}^{-1}$ . The  
65 sample was eluted with a gradient of Milli-Q water (containing 5 mM ammonium formate  
66 with 0.0025% formic acid (both Sigma-Aldrich), eluent A) and methanol (containing 5  
67 mM ammonium formate with 0.0025% formic acid, eluent B) with a flow rate of 0.5 ml  
68  $\text{min}^{-1}$ . Eluent B was increased from 10% to 90% in 10 minutes and maintained for 3  
69 minutes. After this, eluent B was decreased to 10% in 0.1 minute and maintained for 1.9  
70 minute to complete the cycle of 15 minutes. Mass spectrometry was performed with a gas  
71 temperature of 350 $^{\circ}\text{C}$  and a flow rate of 10 l  $\text{min}^{-1}$ . Stealth gas temperature (for Agilent  
72 6460) was set at 400  $^{\circ}\text{C}$  with a flow rate of 12 l  $\text{min}^{-1}$ . The capillary voltage was set at  
73 4000 V.

75 *Direct injection of nylon leachate*

76 An injection volume of 10  $\mu$ l diluted nylon leachate was directly injected into an Agilent  
77 6410 triple quadrupole MS system with ESI in positive SCAN mode. The conditions were  
78 as follows: gas temperature 350 °C, flow rate 10 l min<sup>-1</sup>, mobile phase 50:50 ratio of 80:20  
79 acetonitrile (VWR):Milli-Q water with 5 mM ammonium formate and 10:90  
80 acetonitrile:Milli-Q water with 5 mM ammonium formate, scan range 50–1000 Da,  
81 capillary voltage 3500 V.

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83 **<sup>1</sup>H Nuclear magnetic resonance**

84 The chemical structure of the oligomers was confirmed by <sup>1</sup>H NMR spectroscopy (Bruker  
85 Avance 400 spectrometer). The oligomers were dissolved in ~0.5 ml CD<sub>3</sub>OD:CDCl<sub>3</sub> (1:1)  
86 (Sigma-Aldrich). The spectra were recorded at 24 °C, and internally referenced to the  
87 residual solvent resonance (CD<sub>3</sub>OD: <sup>1</sup>H  $\delta$  3.31).

88 Nylon monomer, yield = 77 mg. <sup>1</sup>H NMR (400.1 MHz, CD<sub>3</sub>OD/CDCl<sub>3</sub> 50:50, 297 K)  $\delta$  =  
89 7.56 (br. m, 2H; NHCO), 3.22 (m, 4H, NHCH<sub>2</sub>), 2.19 (m, 4H, COCH<sub>2</sub>), 1.63 (m, 4H,  
90 COCH<sub>2</sub>CH<sub>2</sub>), 1.54 (m, 4H, NHCH<sub>2</sub>CH<sub>2</sub>), 1.32 (m, 4H, NH (CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>).

91 Nylon dimer, yield = 74 mg. <sup>1</sup>H NMR (400.1 MHz, CD<sub>3</sub>OD/CDCl<sub>3</sub> 50:50, 297 K)  $\delta$  =  
92 7.68 (br. m, 4H; NHCO), 3.15 (t, 8H, NHCH<sub>2</sub>), 2.18 (m, 8H, COCH<sub>2</sub>), 1.60 (m, 8H,  
93 COCH<sub>2</sub>CH<sub>2</sub>), 1.47 (m, 8H, NHCH<sub>2</sub>CH<sub>2</sub>), 1.31 (m, 8H, NH (CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>).

94 Nylon trimer, yield = 16 mg. <sup>1</sup>H NMR (400.1 MHz, CD<sub>3</sub>OD/CDCl<sub>3</sub> 50:50, 297 K)  $\delta$  =  
95 7.74 (br. m, 6H; NHCO), 3.14 (t, 12H, NHCH<sub>2</sub>), 2.18 (m, 12H, COCH<sub>2</sub>), 1.60 (m, 12H,  
96 COCH<sub>2</sub>CH<sub>2</sub>), 1.48 (m, 12H, NHCH<sub>2</sub>CH<sub>2</sub>), 1.32 (m, 12H, NH (CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>).

99 **Supplementary tables and figures**

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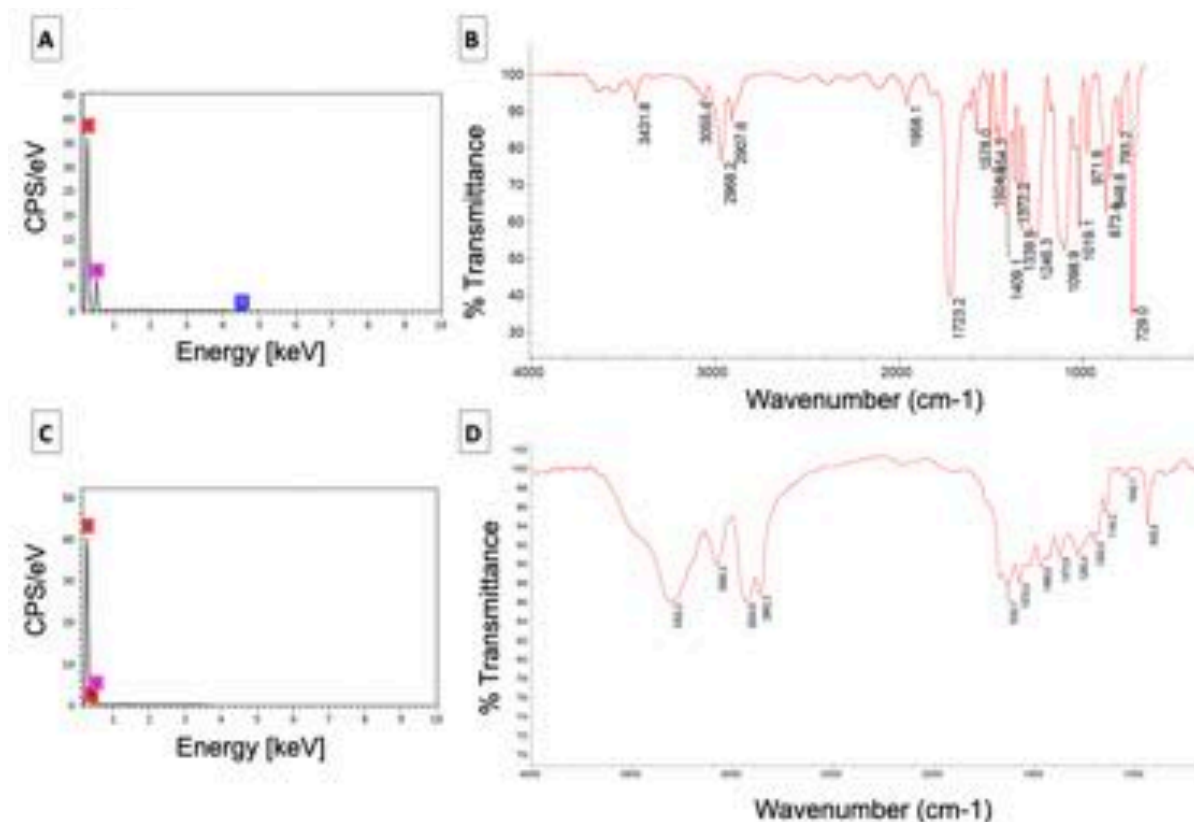
**Table S1.** Size characteristics of polyester and nylon reference microfibers.

	Microfiber size Diameter x length ( $\mu\text{m}$ )	
	Polyester	Nylon
25% percentile	14x50	11x29
Median	15x52	12x31
75% percentile	15x53	12x32
Minimum	13x22	9x24
Maximum	18x64	14x74

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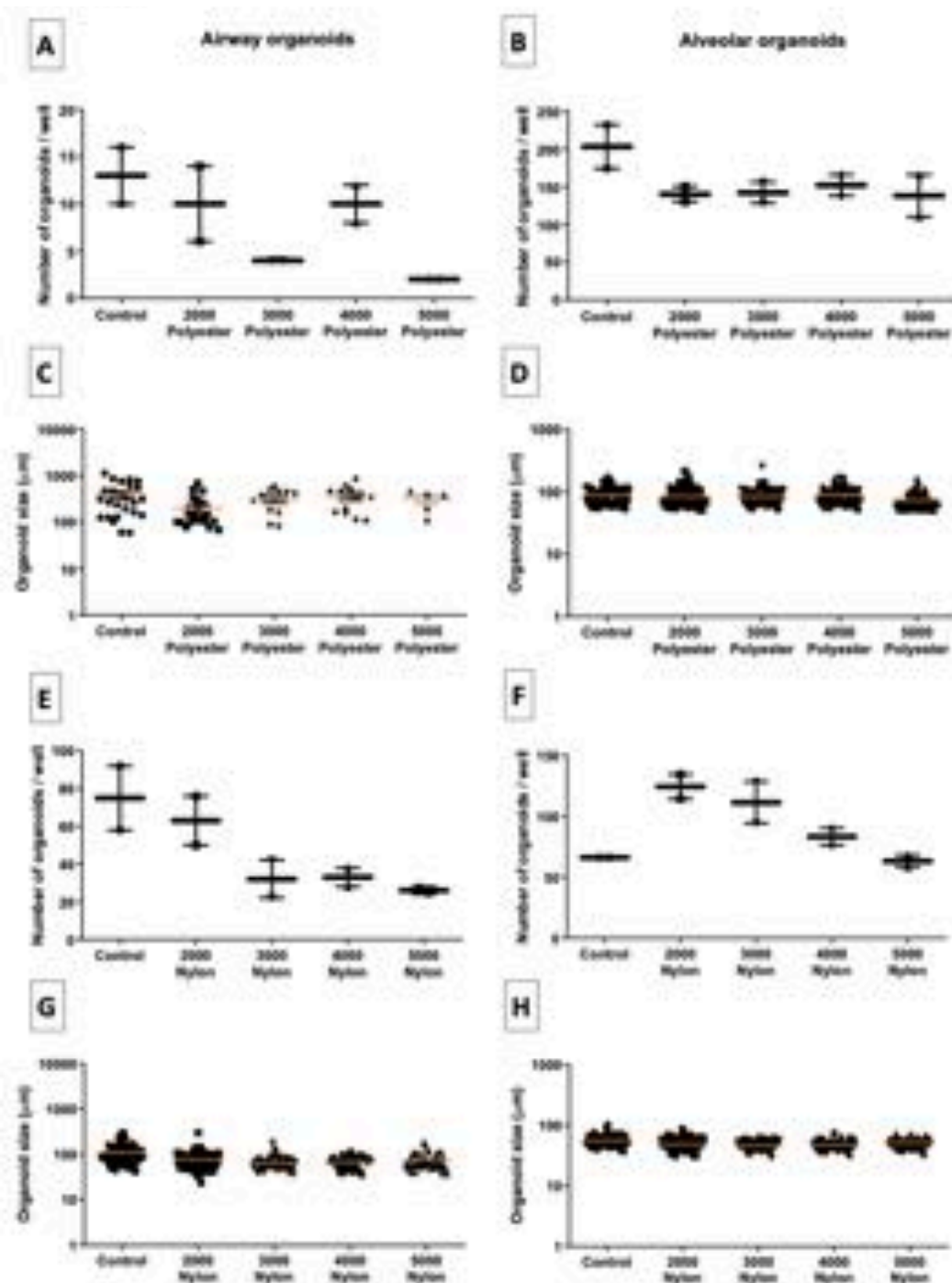
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**Figure S1: Characterization of the reference microfibers using energy dispersive X-ray - and infrared spectroscopy.** (A) EDX spectrum of polyester, confirming the presence of carbon (C) and oxygen (O), and additionally revealing the presence of titanium (Ti), which can be ascribed to small TiO<sub>2</sub> pigment particles used as filler material in these fibers. (B) μFTIR spectrum of polyester with characteristic absorbance peaks (2968 cm<sup>-1</sup>, C-H stretch; 1723 cm<sup>-1</sup>, C=O stretch; 1246 cm<sup>-1</sup>, C-O stretch aromatic ester; 729 cm<sup>-1</sup>, benzene derivative (81)). (C) EDX spectrum of nylon, confirming the presence of carbon (C), nitrogen (N) and oxygen (O). (D) μFTIR spectrum of nylon with characteristic nylon absorbance peaks (3302 cm<sup>-1</sup>, N-H stretch; 2934 cm<sup>-1</sup>, C-H stretch; 1632 cm<sup>-1</sup>, C=O stretch sec. amide; 1202 cm<sup>-1</sup>, C-N bend (81)).



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18 **Figure S2: Determination of the optimal microfiber dose for subsequent in vitro testing**  
19 **of polyester and nylon reference microfibers, using 2000, 3000, 4000 and 5000**  
20 **microfibers per condition. (A, E) Quantification of airway and (B, F) alveolar organoid**  
21 **numbers for organoids exposed to polyester or nylon, respectively (n=2 independent**  
22 **isolations). (C, G) Quantification of the airway and (D, H) alveolar organoid size**  
23 **following exposure to polyester or nylon. 2000, 3000, 4000 or 5000 fibers per well**  
24 **corresponded to approximately 49, 73, 98 and 122  $\mu\text{g/ml}$  of polyester fibers or 16, 23, 31,**  
25 **and 39  $\mu\text{g/ml}$  of nylon fibers.**

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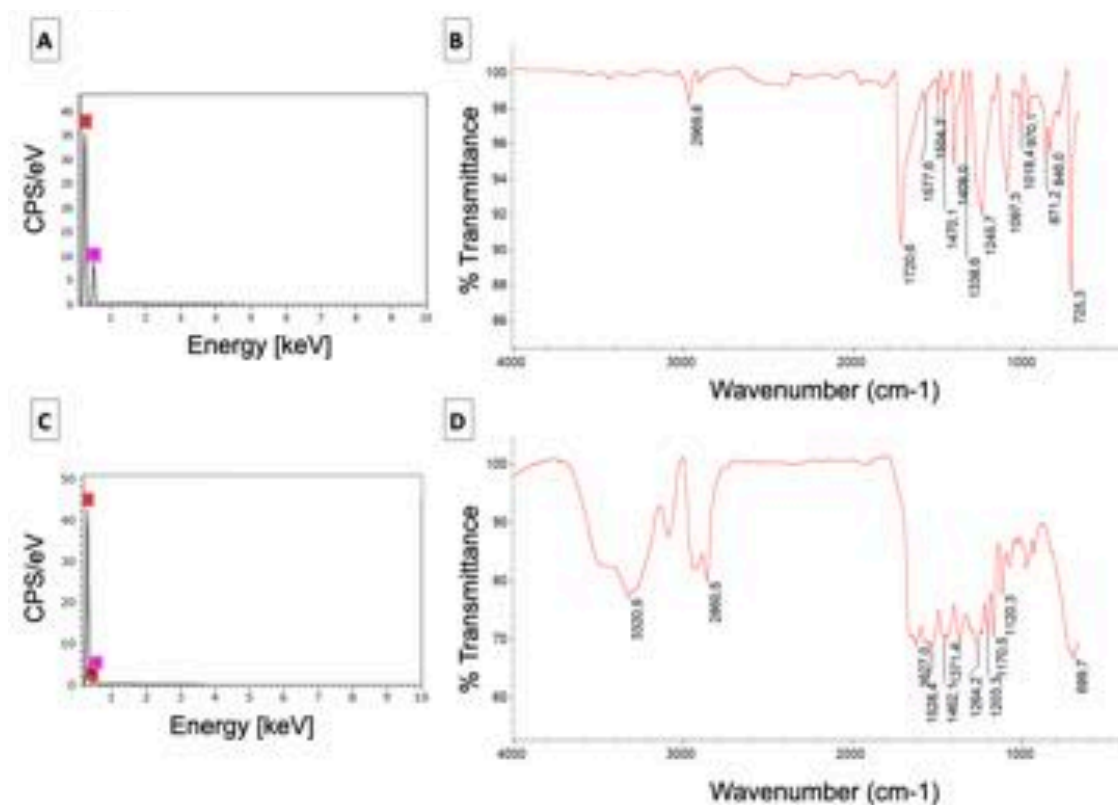
**Table S2.** Size characteristics of polyester and nylon environmental microfibers.

	Microfiber size Diameter x length ( $\mu\text{m}$ )	
	Environmental polyester	Environmental nylon
25% percentile	15x54	46x15
Median	17x63	57x20
75% percentile	18x85	73x27
Minimum	8x30	17x8
Maximum	24x269	296x66

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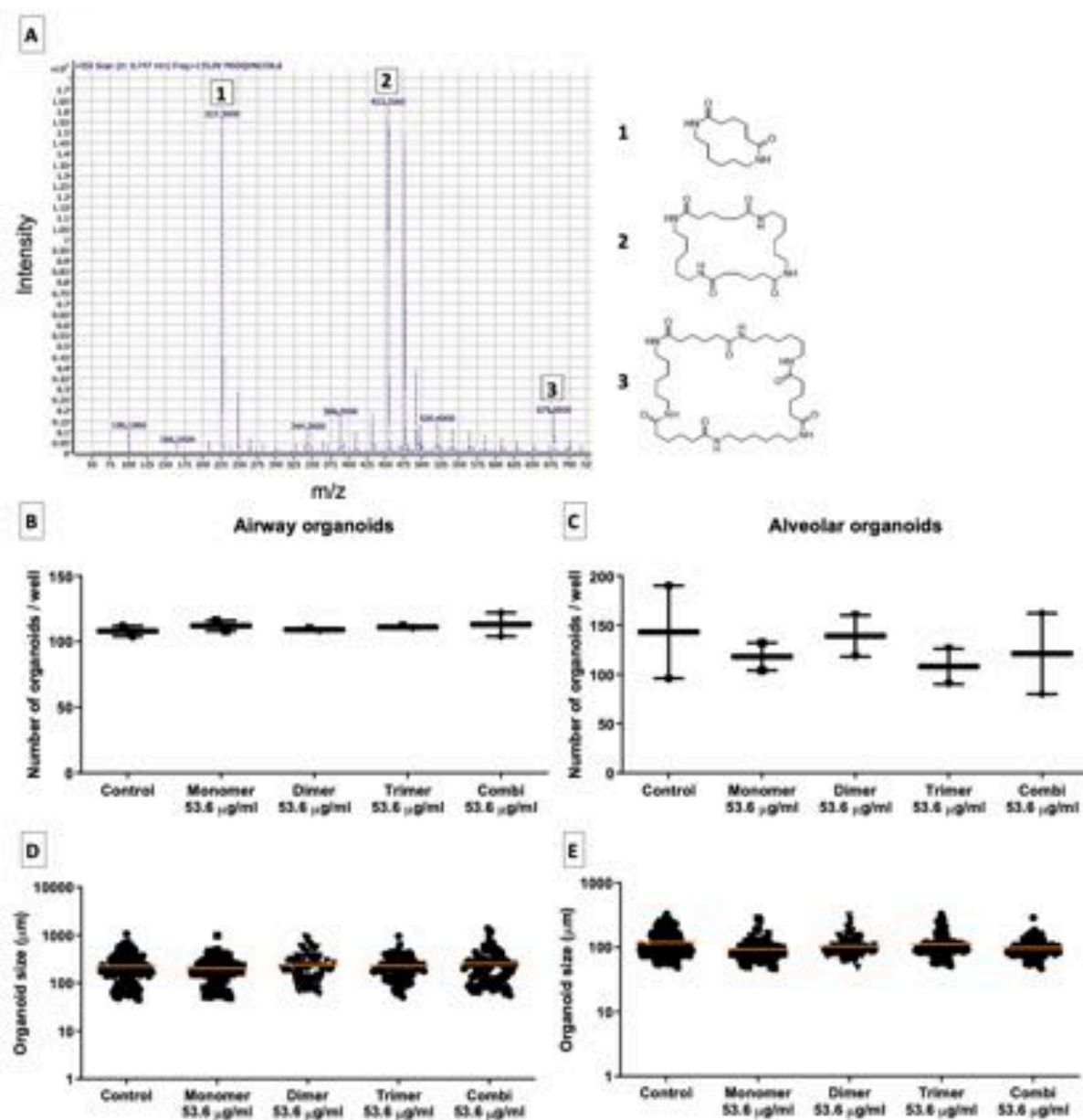
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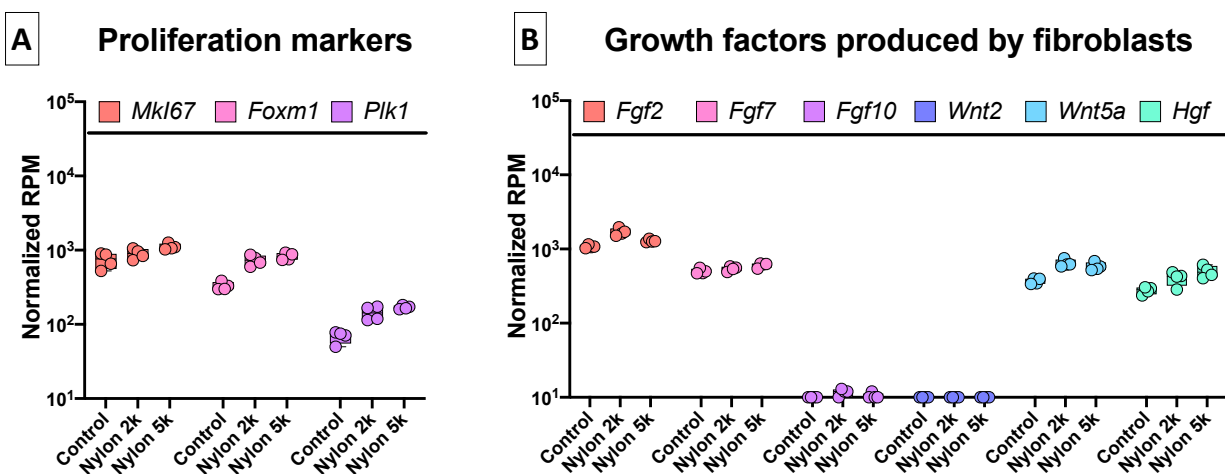
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**Figure S3: Characterization of the environmental microfibers using energy dispersive X-ray - and infrared spectroscopy. EDX - and  $\mu$ -FTIR spectra of (A and B) polyester and (C and D) nylon microfibers.**



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35 **Figure S4: Characterization of the components leaching from nylon reference**  
36 **microfibers and their effect on organoid growth. (A) Mass spectrometry spectrum of the**  
37 **nylon leachate, revealing high amounts of cyclic nylon mono-, di- and trimers, as well as**  
38 **other smaller peaks. (B and C) Assessment of the numbers of airway and alveolar**  
39 **organoids and (D and E) their sizes (n=2 independent isolations).**





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**Figure S5: Expression of individual genes in fibroblasts from organoid cultures exposed to nylon microfibers. (A) Genes associated with proliferation of fibroblasts. (B) Genes encoding factors produced by fibroblasts important for epithelial development (n=4 independent isolations).**