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Improving Pinched Flow Fractionation through Hybrid Inlet Design for Label-Free Size-Based Separation of Microalgae

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Abstract

Efficient, label-free separation of microalgae is essential for environmental monitoring, algal cultivation, and downstream biological analysis. Although pinched flow fractionation (PFF) is a promising passive microfluidic technique for size-based separation, its performance is often compromised by particle retention and unstable hydrodynamic focusing near the sample inlet. In this study, we present a **hybrid-inlet PFF microfluidic chip** designed to overcome these limitations while preserving the simplicity of conventional PFF.

A two-dimensional COMSOL model coupling laminar flow and particle tracing was established to investigate flow characteristics and optimize inlet flow-rate ratios. Three inlet configurations, including partially opened, fully opened, and hybrid designs, were systematically compared. The hybrid inlet effectively reduced particle retention while maintaining stable particle focusing, resulting in improved separation reproducibility.

The optimized device was first validated using 3 and 15 μm polystyrene microparticles and subsequently applied to separate two representative microalgal species, *Nannochloropsis* sp. (3–5 μm) and *Thalassiosira weissflogii* (11–16 μm). Under an optimized inlet flow-rate ratio of 1:9, sorting rates reached **90.3%** and **92.0%**, with purities of **96.2%** and **92.7%**, respectively, while recovery rates exceeded **96%** for both species. Four independent experiments further demonstrated excellent repeatability, with sorting-rate variations below 1%.

These results demonstrate that the proposed hybrid-inlet architecture substantially improves the robustness of PFF by minimizing inlet-induced particle loss while maintaining high separation efficiency. The presented platform provides a simple, passive, and reproducible strategy for label-free size-based separation of microalgae and other biological particles.

Keywords: Pinched flow fractionation; Microfluidic chip; Microalgae separation; Size-based separation; Passive separation

1. Introduction

Microalgae are photosynthetic microorganisms with considerable ecological and industrial importance (Metting 1996, Singh and Saxena 2015). They contribute substantially to aquatic primary production and carbon fixation and have been widely investigated for applications in biofuels, wastewater treatment, carbon capture, food supplements, pharmaceuticals, and the production of high-value compounds (Brennan and Owende 2010, M. U, Mehar et al. 2019, Mutanda, Naidoo et al. 2020, Maltsev and Maltseva 2021, Viswanaathan, Perumal et al. 2022). The isolation and purification of specific microalgal species are therefore essential for environmental analysis, strain cultivation, biological research, and downstream bioprocessing. However, natural microalgal samples commonly contain cells with different sizes, morphologies, densities, and surface properties, together with suspended particles and cellular debris, making efficient separation difficult (Gonzalez de Vega, Goyen et al. 2021).

Conventional microalgal separation methods include centrifugation, membrane filtration, sedimentation, manual micropipette isolation, and flow cytometry. Centrifugation and filtration are relatively straightforward and can process large sample volumes, but they may require substantial energy consumption, cause cell damage, or exhibit limited selectivity when the target cells have similar physical properties. Manual isolation under a microscope provides high accuracy but is time-consuming, operator-dependent, and unsuitable for high-throughput processing. Flow cytometry enables precise multiparametric analysis and cell sorting; however, the equipment is costly, requires trained personnel, and may not be practical for routine or field-based applications (Čertnerová and Galbraith 2021). These limitations have motivated the development of compact, low-cost, and label-free separation platforms (Gossett, Weaver et al. 2010).

Microfluidic technology provides precise manipulation of small sample volumes and has emerged as a promising approach for particle and cell separation (Verpoorte and Rooij 2003, Zhang, Yuan et al. 2017). Microfluidic separation methods can generally be divided into active and passive techniques. Active methods use external electric, magnetic, acoustic, optical, or thermal fields to control particle motion (Nedelcu, Corman et al. 2015, Wang, Ren et al. 2017, Chen, Huang et al. 2022, Kalyuzhnaya, Sokolov et al. 2023). Although these methods offer high controllability and selectivity, they require additional equipment and increase system complexity and

operating cost. In contrast, passive microfluidic methods rely primarily on channel geometry and hydrodynamic effects and therefore offer simpler structures, lower energy consumption, and easier integration (Mansor, Jamrus et al. 2025). These characteristics make passive systems particularly attractive for biological sample pretreatment and portable analysis.

To clarify the rationale for selecting the separation method used in this study, the characteristics of conventional and microfluidic separation techniques are compared in Table 1.

Table 1. Comparison of conventional and microfluidic methods for microalgal separation

Method	Separation basis	Advantages	Limitations	Suitability for this study
Centrifugation	Density / sedimentation	Large sample volume	Cell damage, high energy, limited selectivity	Low
Membrane filtration	Cell size	Simple operation	Clogging and cell retention	Moderate
Flow cytometry	Optical signals	High selectivity	Expensive and complex equipment	Low
Active microfluidics	Electric, acoustic or magnetic fields	High controllability	Requires external equipment	Moderate
Passive PFF	Hydrodynamic size displacement	Label-free, simple, low energy	Mainly dependent on size	High

As summarized in Table 1, conventional separation methods are suitable for large-volume processing but may suffer from limited selectivity, cell damage, or high equipment requirements. Active microfluidic methods provide precise manipulation but require additional external components. In contrast, passive PFF enables continuous, label-free, and size-based separation through channel geometry and hydrodynamic control. Therefore, PFF was selected as the fundamental separation mechanism in this study.

Pinched flow fractionation (PFF) is a passive size-based separation technique in which a sample flow is hydrodynamically compressed against a channel wall by a

sheath flow in a narrow pinched segment. Because the centers of particles with different diameters occupy different lateral positions near the wall, the particles follow spatially separated streamlines after entering an expanded region and can subsequently be collected through different outlets. PFF devices have been applied to the separation of polymer particles, cells, and other biological samples because they do not require particle labeling or external actuation. Their separation performance can be adjusted through the flow-rate ratio, pinched-channel dimensions, expansion geometry, and outlet configuration.

Nevertheless, several challenges remain when PFF devices are applied to microalgal samples. Many previous studies have primarily used standardized spherical microparticles or individual biological cell types (Yamada, Nakashima et al. 2004, Funakoshi, Akiyama et al. 2011), whereas actual microalgae may exhibit irregular shapes, broad size distributions, surface adhesion, and aggregation. These biological characteristics can alter particle trajectories and cause retention or blockage within the microchannel. In addition, the inlet-opening geometry and tubing connection may generate local flow disturbances or recirculation, resulting in unstable particle focusing and mis-sorting. Particle adhesion in the inlet, channel, outlet, and collection tubing may further reduce recovery and experimental repeatability. Therefore, inlet design and sample-delivery conditions must be considered together with the main separation geometry when developing a reliable PFF platform for microalgae.

In this study, a modified-inlet PFF microfluidic chip was developed for the passive size-based separation of microparticles and microalgae. A two-dimensional numerical model combining laminar flow and particle tracing was established using COMSOL Multiphysics to investigate the flow-field distribution and particle trajectories under different inlet flow-rate ratios. Different inlet-opening configurations, including partially opened, fully opened, and hybrid inlet designs, were experimentally evaluated to reduce particle retention while maintaining stable hydrodynamic focusing. The outlet tubing length and surfactant concentration were also adjusted to improve particle collection and recovery. The proposed device was initially validated using 3 and 15 μm polystyrene microparticles and was subsequently applied to two microalgal species with distinct size ranges, namely *Nannochloropsis* sp. and *Thalassiosira weissflogii*. Separation performance was quantitatively assessed using sorting rate, mis-sorting rate, purity, recovery rate, and repeatability. The results demonstrate the potential of the

modified PFF chip as a simple and reproducible platform for microalgal separation and biological sample pretreatment. The overall research concept, target samples, and size-based separation principle of the proposed PFF microfluidic platform are summarized in Fig. 1.

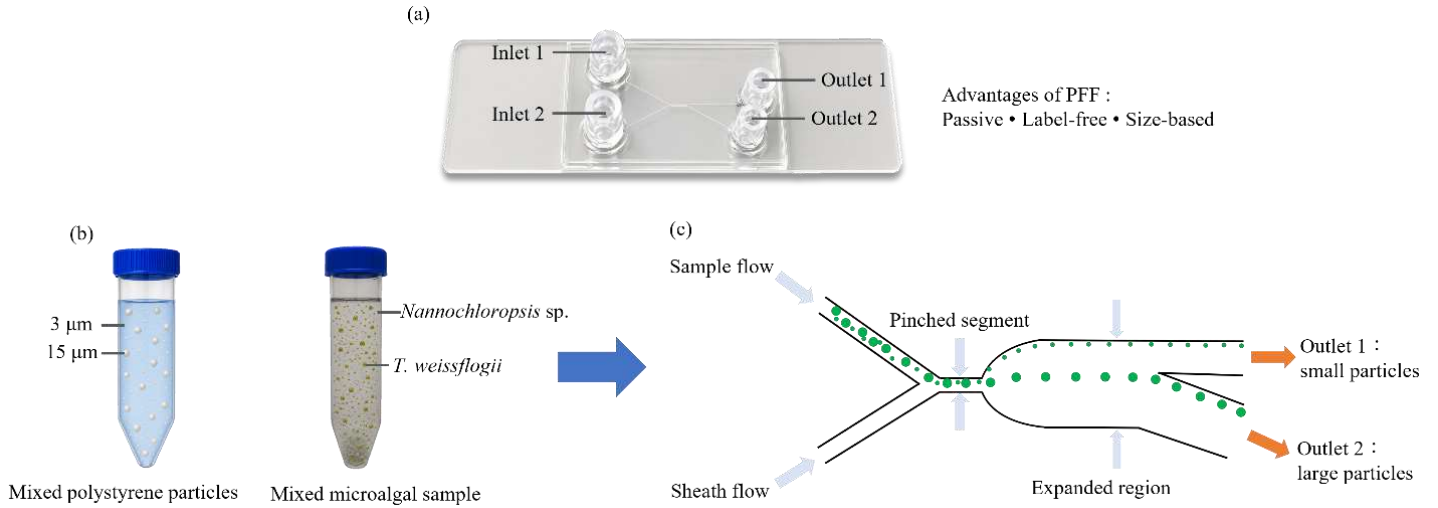


Fig. 1 Overview of the passive, label-free PFF microfluidic platform for size-based separation of microparticles and microalgae: (a) fabricated chip with two inlets and two outlets; (b) representative mixed polystyrene-particle and microalgal samples; and (c) hydrodynamic focusing and size-dependent particle displacement in the pinched and expanded regions, resulting in collection through different outlets.

1. Materials and methods

2.1. Microfluidic Chip Design and Numerical Simulation

The microfluidic chip was designed based on the principle of pinched flow fractionation (PFF). The device consisted of two inlets, a pinched segment, an expanded separation region, and two outlets. Inlet 1 was used to introduce the particle or microalgal suspension, whereas Inlet 2 supplied the sheath flow. The two streams converged at the pinched segment, where the sample stream was hydrodynamically compressed against the lower channel wall.

The width of the pinched segment was 50 μm , and the downstream expanded region was divided into Outlet 1 and Outlet 2 with channel widths of 200 and 520 μm , respectively. Because the centers of larger particles were located farther from the channel wall than those of smaller particles, particles of different sizes followed different streamlines after the expansion. Smaller particles were primarily directed toward Outlet 1, whereas larger particles were collected from Outlet 2.

Three inlet-opening configurations were evaluated: a partially opened inlet, a fully opened inlet, and a hybrid inlet. The partially opened configuration restricted the opening to the lower region of Inlet 1, whereas the fully opened configuration retained the complete inlet opening. The hybrid configuration combined the geometric features of the two designs.

A two-dimensional numerical model was established using COMSOL Multiphysics to analyze the flow-field distribution and particle trajectories in the microfluidic channel (Slay, Mancinelli et al. 2024). The Laminar Flow and Particle Tracing for Fluid Flow modules were coupled in the simulation. Deionized water was used as the working fluid, with a density of 1000 kg/m^3 and a dynamic viscosity of $0.001 \text{ Pa}\cdot\text{s}$. The channel walls were assigned a no-slip boundary condition, and the pressures at both outlets were set to zero gauge pressure.

The volumetric flow rates at Inlet 1 and Inlet 2 were independently specified to investigate the influence of the inlet flow-rate ratio. For the polystyrene-particle simulations, particles with diameters of 3 and $15 \text{ }\mu\text{m}$ were introduced into the sample stream, and flow-rate ratios of $1:1$ and $1:6$ were evaluated. For the microalgae simulations, equivalent spherical particle diameters of $3\text{--}5 \text{ }\mu\text{m}$ and $12\text{--}15 \text{ }\mu\text{m}$ were used to represent *Nannochloropsis* sp. and *Thalassiosira weissflogii*, respectively. Flow-rate ratios of $1:1$ and $1:9$ were compared to determine the conditions required for size-dependent trajectory separation.

The computational domain was discretized using triangular elements. A refined mesh was applied to the pinched segment and the expansion region because these regions exhibited relatively large velocity gradients and considerable changes in particle trajectories. A coarser mesh was applied to the remaining channel regions to reduce computational cost while maintaining numerical accuracy. The chip geometry, channel dimensions, and simulated velocity field are shown in Fig. 2.

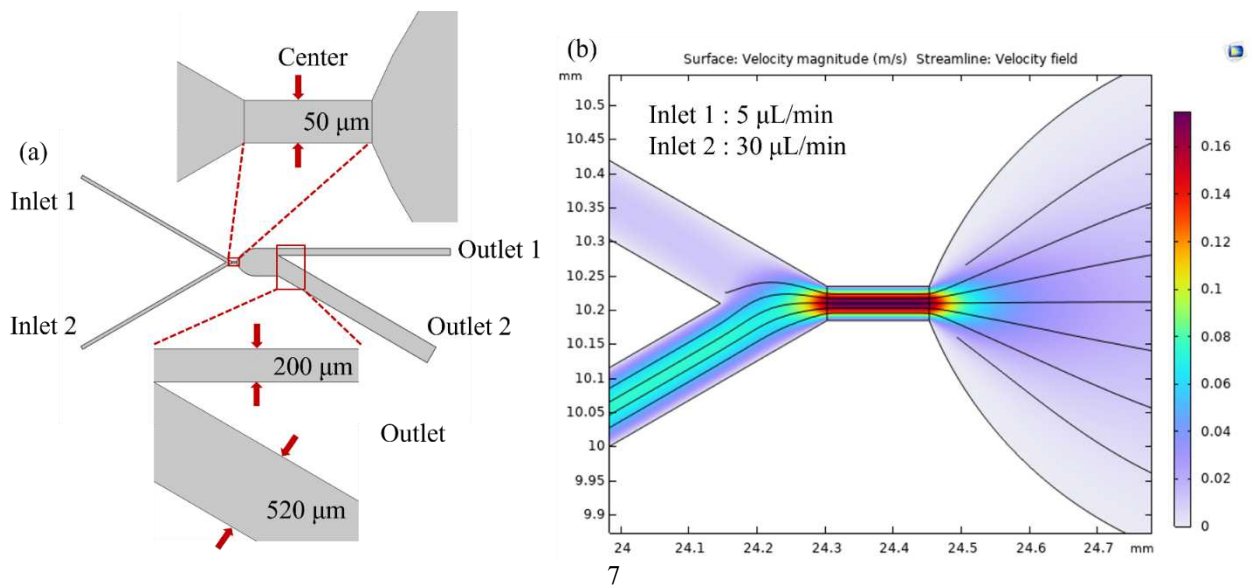


Fig. 2 Microfluidic chip design and simulated flow field of the PFF device: (a) chip geometry and channel dimensions, and (b) simulated velocity magnitude and streamlines at Inlet 1 and Inlet 2 flow rates of 5 and 30 $\mu\text{L}/\text{min}$, respectively.

2.2. Microfluidic chip fabrication

The master mold was fabricated on a silicon wafer using standard photolithography. SU-8 3050 negative photoresist was spin-coated onto the substrate to obtain a target channel height of approximately 50 μm . After soft baking, ultraviolet exposure through a patterned photomask, post-exposure baking, and development, an SU-8 microchannel structure was formed on the silicon substrate. The thickness of the patterned SU-8 layer was measured using an Alpha-Step surface profilometer, while the widths of the microchannel features were measured using a VHX-7000 digital microscope. The characterized SU-8 structure was subsequently used as the master mold for polydimethylsiloxane replication.

Polydimethylsiloxane (PDMS) is widely used in microfluidic device fabrication because of its optical transparency, biocompatibility, and ease of replication (Fanous, Ozhikandathil et al. 2015). The PDMS prepolymer and curing agent were mixed at a weight ratio of 10:1. The mixture was degassed under vacuum for 20 min to remove trapped air bubbles and subsequently poured onto the SU-8 master mold. The PDMS was cured at 95 $^{\circ}\text{C}$ for 50 min and carefully peeled from the master mold after cooling. The replicated PDMS layer was then cut to the required dimensions, and the inlet and outlet openings were formed according to the selected inlet configuration.

The patterned PDMS layer and a glass substrate were treated with oxygen plasma and immediately brought into contact to achieve irreversible PDMS–glass bonding. The bonded device was subsequently heated at 95 $^{\circ}\text{C}$ for 30 min to improve its bonding strength and long-term stability.

2.3. Preparation of microparticle and microalgae samples

Polystyrene microparticles with nominal diameters of 3 and 15 μm were used to evaluate the basic separation performance of the microfluidic chip. The particle suspensions were prepared in deionized water. Tween 20 was added at a concentration of 0.0001% to reduce particle adhesion to the inlet, microchannel walls, outlet regions, and collection tubing.

Single-particle suspensions were first used to determine the trajectories and appropriate operating conditions for each particle size. Mixed-particle suspensions were subsequently prepared using both 3 and 15 μm particles. For purity evaluation, samples containing approximately equal initial numbers of the two particle sizes were used to prevent the initial particle-number ratio from dominating the outlet purity.

Two microalgal species with different size ranges were selected for biological validation. *Nannochloropsis* sp. consisted primarily of approximately spherical cells with diameters of 3–5 μm , whereas *Thalassiosira weissflogii* exhibited larger cells with characteristic dimensions of approximately 11–16 μm . Single-species experiments were first performed, followed by separation experiments using mixtures of the two microalgal species.

2.4. Experimental setup and separation procedure

The experimental system consisted of two syringe pumps, an inverted optical microscope, an image-acquisition device, the microfluidic chip, and outlet collection tubes. Inlet 1 was connected to a syringe containing the particle or microalgal suspension, whereas Inlet 2 was connected to a syringe containing the sheath fluid. Both fluids were continuously introduced into the microfluidic chip using independently controlled syringe pumps.

The experimental setup and sample-collection procedure are illustrated in Fig. 3.

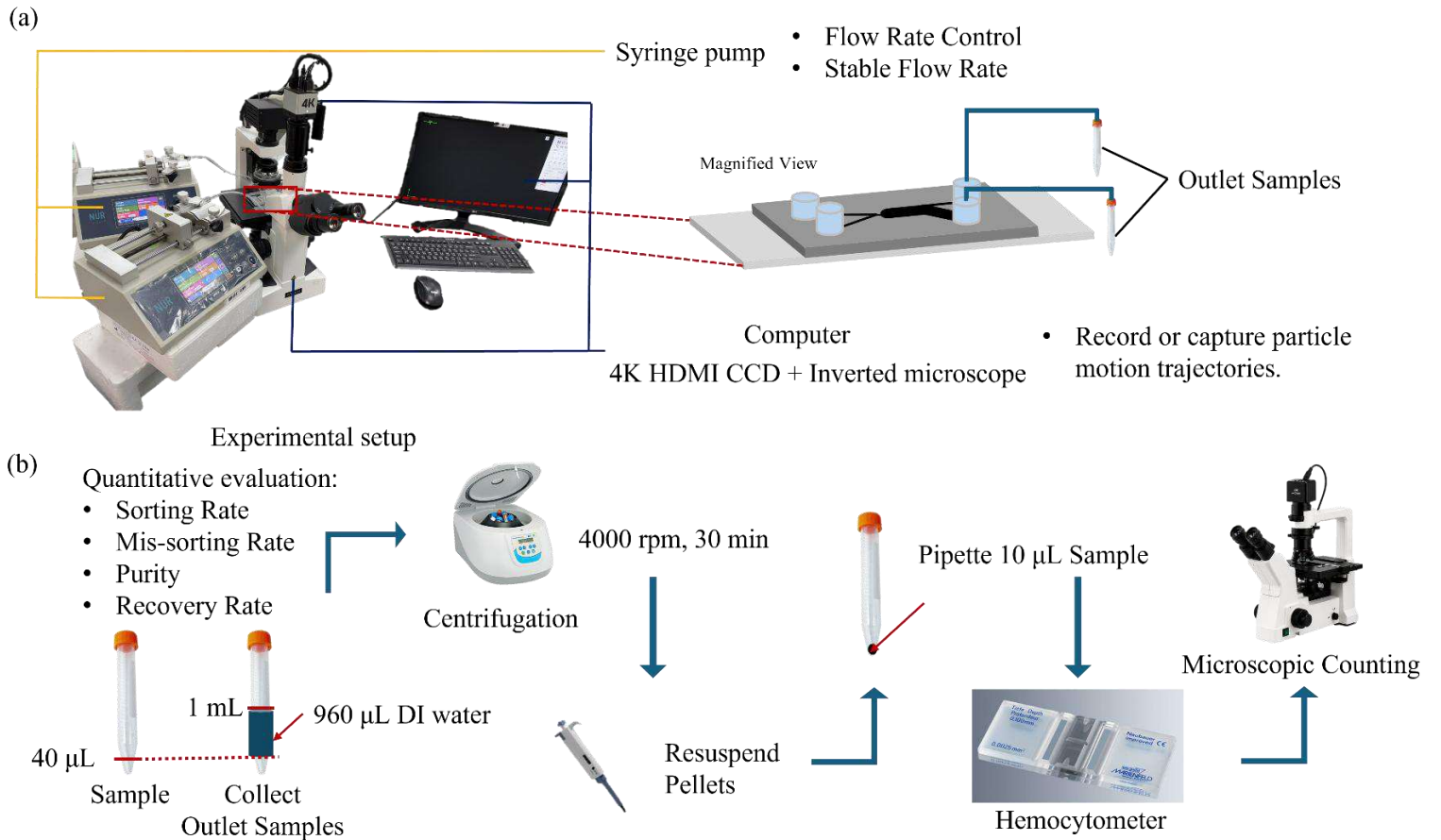


Fig. 3 Experimental setup and quantitative analysis procedure: (a) The experimental setup consisting of two syringe pumps, an inverted microscope, an imaging system, and outlet collection tubes; and (b) sample collection and particle-counting procedure.

After separation, 40 μL of each outlet sample was diluted with 960 μL of deionized water to a final volume of 1 mL. The samples were subsequently centrifuged at 4000 rpm for 30 min. After removing a portion of the supernatant, the pellets were resuspended, and 10 μL aliquots were loaded into a hemocytometer for counting.

For the final polystyrene-particle experiments using the hybrid inlet design, the flow rates of Inlet 1 and Inlet 2 were set to 10 and 100 $\mu\text{L}/\text{min}$, respectively, corresponding to a flow-rate ratio of 1:10. The final microalgae separation experiments were conducted at flow rates of 10 and 90 $\mu\text{L}/\text{min}$, corresponding to a flow-rate ratio of 1:9. The flow-rate ratio for microalgae was adjusted because the broad size distribution and nonspherical morphology of *T. weissflogii* resulted in different trajectories from those of the standardized polystyrene microparticles.

The length of the tubing connected to Outlet 2 was shortened to three-quarters of its original length to reduce hydraulic resistance and improve the collection of larger particles. Samples discharged from Outlet 1 and Outlet 2 were collected separately for subsequent quantitative analysis. Particle and cell trajectories within the microchannel were recorded using the inverted microscope and image-acquisition system.

2.5. Quantitative evaluation of separation performance

Before each separation experiment, the sample suspension was thoroughly mixed, and a 10 μL aliquot was loaded into a hemocytometer. The initial numbers of each particle type or microalgal species were counted using an inverted microscope.

After separation, the samples collected from Outlet 1 and Outlet 2 were centrifuged at 4000 rpm for 30 min. A portion of the supernatant was removed, and the concentrated samples were resuspended before counting. Each sample was measured repeatedly to reduce counting variability.

The separation performance was evaluated using the sorting rate, mis-sorting rate, purity, and recovery rate. The sorting rate was defined as

$$\text{Sorting Rate(\%)} = \frac{N_{\text{correct}}}{N_{\text{initial}}} \times 100\% \quad (1)$$

where N_{correct} is the number of target particles collected from the intended outlet and N_{initial} is the initial number of the corresponding particle type.

The mis-sorting rate was calculated as

$$\text{Mis-sorting Rate(\%)} = \frac{N_{\text{incorrect}}}{N_{\text{initial}}} \times 100\% \quad (2)$$

where $N_{\text{incorrect}}$ is the number of target particles collected from the unintended outlet.

For mixed samples, the purity of each intended outlet was calculated as

$$\text{Purity}(\%) = \frac{N_{\text{target,outlet}}}{N_{\text{total,outlet}}} \times 100\% \quad (3)$$

where $N_{\text{target,outlet}}$ represents the number of target particles in the selected outlet and $N_{\text{total,outlet}}$ represents the total number of particles collected from the same outlet.

The recovery rate was defined as

$$\text{Recovery Rate}(\%) = \frac{N_{\text{outlet 1}} + N_{\text{outlet 2}}}{N_{\text{initial}}} \times 100\% \quad (4)$$

where $N_{\text{outlet 1}}$ and $N_{\text{outlet 2}}$ are the numbers of the corresponding particle type collected from the two outlets.

2.6. Repeatability analysis

The separation experiments were repeated under identical operating conditions to evaluate the stability and repeatability of the proposed device. At least three measurements were performed for individual experimental conditions. Four repeated experiments were conducted for the mixed-microalgae separation, and the results were reported as the mean $\pm$ standard deviation.

2. Results and Discussion

3.1. Numerical analysis of particle trajectories

The flow-field and particle-tracing simulations were performed to investigate the size-dependent separation behavior of the proposed PFF microfluidic chip. After the sample flow and sheath flow entered the pinched segment, the higher sheath-flow rate compressed the sample stream toward the lower channel wall. The particle centers were consequently distributed at different lateral positions according to their diameters. After the particles entered the expanded region, these differences in lateral position were amplified, causing particles of different sizes to follow distinct streamlines toward the two outlets.

For the polystyrene microparticle model, the trajectories of 3 and 15 μm particles were first investigated. At a relatively low sheath-to-sample flow-rate ratio, the sample stream was insufficiently compressed, and the trajectories of the two particle sizes remained relatively close after the expansion. Increasing the flow-rate ratio strengthened hydrodynamic focusing in the pinched segment. At a flow-rate ratio of 1:6, the 3 μm particles primarily followed the upper streamlines toward Outlet 1, whereas the 15 μm particles followed the lower streamlines toward Outlet 2, as shown in Fig.

4(c). This result was consistent with the fundamental PFF mechanism, in which the distance between the particle center and the channel wall increases with particle diameter. Because the inlet geometry was subsequently modified, the flow-rate ratio was experimentally reoptimized, and a ratio of 1:10 was selected for the final polystyrene-particle separation experiments.

The same trend was observed in the simulations representing microalgal cells. Equivalent spherical diameters of 3–5 μm and 12–15 μm were assigned to *Nannochloropsis* sp. and *Thalassiosira weissflogii*, respectively. At a flow-rate ratio of 1:1, the sheath-flow compression was relatively weak, and the particle trajectories showed insufficient spatial separation. When the flow-rate ratio was increased to 1:9, the smaller particles were concentrated near the upper streamline leading to Outlet 1, whereas the larger particles were directed toward Outlet 2. The mixed-particle simulation therefore predicted that the two microalgal size groups could be separated using the proposed channel geometry. The simulated trajectories of polystyrene microparticles and microalgae-equivalent particles under different inlet flow-rate ratios are compared in Fig. 4.

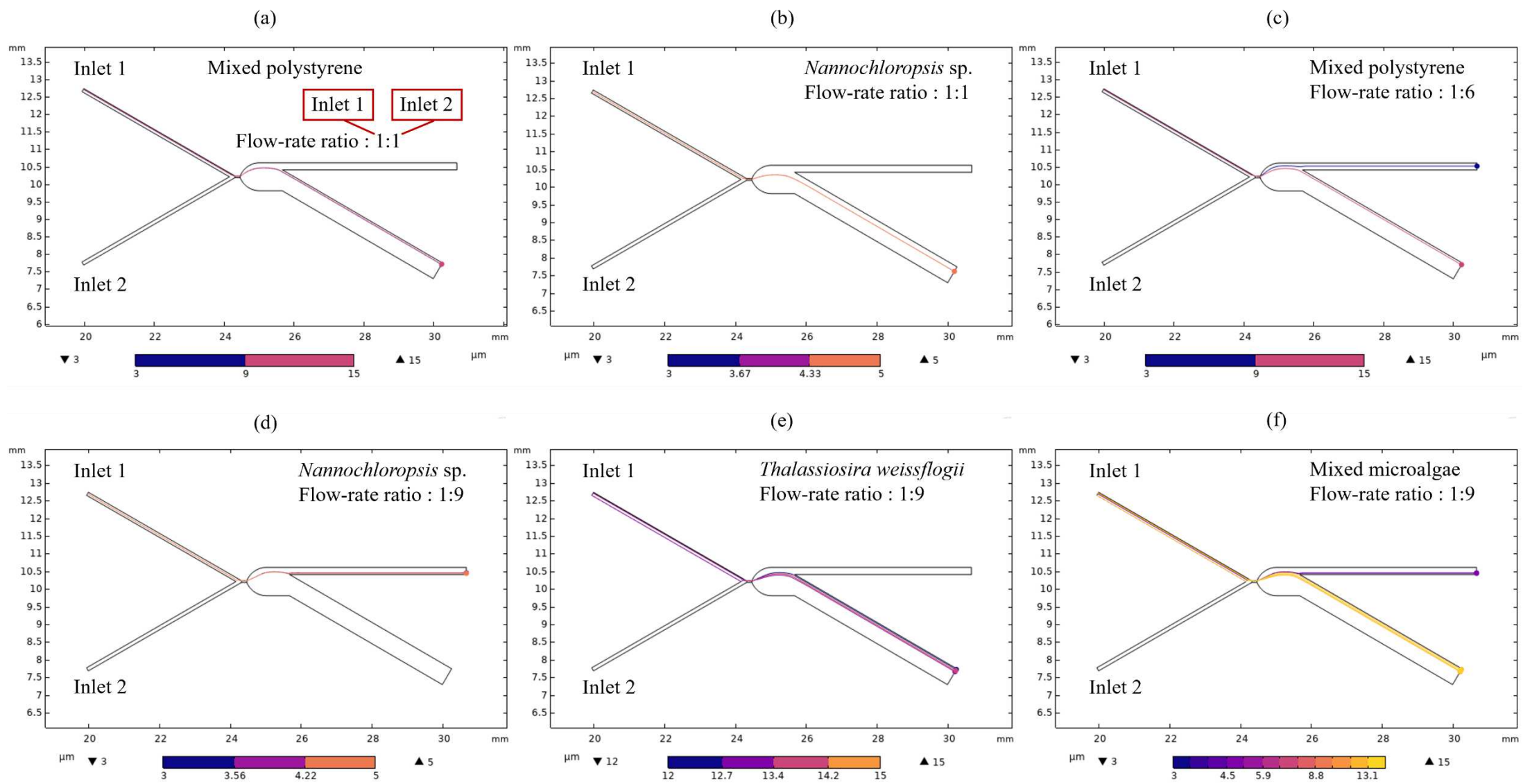


Fig. 4 Simulated particle trajectories under different inlet flow-rate ratios: (a) mixed 3 and 15 μm polystyrene particles at a flow-rate ratio of 1:1; (b) 3–5 μm equivalent particles representing *Nannochloropsis* sp. at 1:1; (c) mixed 3 and 15 μm polystyrene particles at 1:6; (d) 3–5 μm equivalent particles representing *Nannochloropsis* sp. at 1:9; (e) 12–15 μm equivalent particles representing *Thalassiosira weissflogii* at 1:9; and (f) mixed microalgae-equivalent particles at 1:9.

The agreement between the simulated trajectories and the subsequent experimental observations indicates that the numerical model captured the principal hydrodynamic behavior of the device. Nevertheless, the biological cells exhibited broader trajectory distributions than the idealized particles. This difference was attributed to variations in cell size and morphology, particularly for *T. weissflogii*, which was not perfectly spherical and had a wider size distribution. Therefore, the simulation was primarily used to predict the overall separation tendency and determine an appropriate range of operating conditions rather than to reproduce every individual cell trajectory. The simulation results showed that the 3–5 μm equivalent particles were mainly directed to Outlet 1, whereas the 12–15 μm particles were directed to Outlet 2 at a flow-rate ratio of 1:9.

Based on the numerical results, the experimental optimization was subsequently performed using single-size polystyrene particles. The 3 and 15 μm particles were first examined separately to determine the operating conditions required to direct each particle population toward its intended outlet.

2.2. Optimization of single-particle separation conditions

Before the hybrid inlet configuration was adopted, preliminary single-particle experiments were conducted to determine the operating conditions required to direct the 3 and 15 μm polystyrene particles toward their intended outlets. The flow rate of Inlet 1 was fixed at 5 $\mu\text{L}/\text{min}$, while the flow rate of Inlet 2 was sequentially increased from 5 to 30 $\mu\text{L}/\text{min}$, corresponding to flow-rate ratios of 1:1, 1:2, 1:4, and 1:6. The tested flow-rate conditions and the corresponding particle behaviors are summarized in Table 2.

Table 2. Optimization of flow-rate conditions and measured trajectories of single polystyrene particles

Particle size	Inlet 1 ($\mu\text{L}/\text{min}$)	Inlet 2 ($\mu\text{L}/\text{min}$)	Flow-rate ratio	Reference boundary	Measured trajectory position relative to the reference boundary
3 μm	5	5	1:1	Upper wall	161.7 μm from the upper wall
	5	10	1:2		81.0 μm from the upper wall
	5	20	1:4		35.0 μm from the upper wall
	5	30	1:6	—	Directed toward Outlet 1; no particles observed in Outlet 2
15 μm	5	5	1:1	Lower wall	296.7–332.2 μm from the lower wall
	5	10	1:2		383.2–421.6 μm from the lower wall
	5	20	1:4		430.4–466.5 μm from the lower wall
	5	30	1:6	—	Close to the upper boundary of the channel leading to Outlet 2

For the 3 μm particles, increasing the sheath-flow rate progressively shifted the particle trajectories toward Outlet 1. The measured distance between the particle trajectory and the upper wall decreased from 161.7 μm at a flow-rate ratio of 1:1 to 81.0 μm at 1:2 and 35.0 μm at 1:4. At a flow-rate ratio of 1:6, the particles were directed toward Outlet 1, and no particles were visually observed entering Outlet 2 during the observation period. Therefore, 1:6 was identified as the minimum tested flow-rate ratio required for stable collection of the 3 μm particles through Outlet 1.

After modifying the inlet opening and shortening the tubing connected to Outlet 2, the same flow-rate conditions were evaluated using single 15 μm particles. Increasing the sheath-flow rate progressively displaced the particle trajectories away from the lower wall. The measured trajectory range increased from 296.7–332.2 μm at a flow-rate ratio of 1:1 to 383.2–421.6 μm at 1:2 and 430.4–466.5 μm at 1:4. At a flow-rate ratio of 1:6, the particles moved close to the upper boundary of the channel leading to Outlet 2, indicating stable transport toward the intended outlet.

Therefore, 1:6 was identified as the minimum tested flow-rate ratio that provided stable collection of the smaller particles through Outlet 1 while maintaining stable collection of the 15 μm particles through Outlet 2. However, because the inlet geometry was subsequently changed to the hybrid configuration, the flow-rate ratio was reevaluated before the final mixed-particle separation experiments.

2.3. Reoptimization of the Flow-Rate Ratio for the Hybrid Inlet

Among the three inlet configurations, the partially opened inlet improved particle guidance but caused considerable particle retention. The fully opened inlet reduced retention but resulted in greater variation in the initial particle position. The hybrid inlet provided a balance between sample transport and focusing stability and was therefore selected for the final separation experiments. Because modification of the inlet geometry altered the initial particle position and local flow distribution, the flow-rate ratio was reevaluated after the hybrid inlet was introduced. At ratios of 1:1 and 1:6, residual 3 μm particles were still observed in Outlet 2. At 1:10, almost no 3 μm particles entered Outlet 2. Therefore, a flow-rate ratio of 1:10 was selected for the final mixed-particle experiments using the hybrid inlet.

3.4. Separation performance of polystyrene microparticles

After the operating conditions had been evaluated using single-size particles and the flow-rate ratio had been reoptimized for the hybrid inlet, the separation performance of the device was evaluated using a mixed suspension of 3 and 15 μm polystyrene microparticles. Approximately equal initial numbers of the two particle populations were used to minimize the influence of the initial particle-number ratio on the calculated outlet purity. The experiments were conducted at Inlet 1 and Inlet 2 flow rates of 10 and 100 $\mu\text{L}/\text{min}$, respectively, corresponding to a flow-rate ratio of 1:10.

Under the optimized operating condition, the 3 μm particles were primarily directed toward Outlet 1, whereas the 15 μm particles followed trajectories leading toward Outlet 2. Representative particle trajectories and the measured trajectory ranges at different locations in the expanded separation region are shown in Fig. 5.

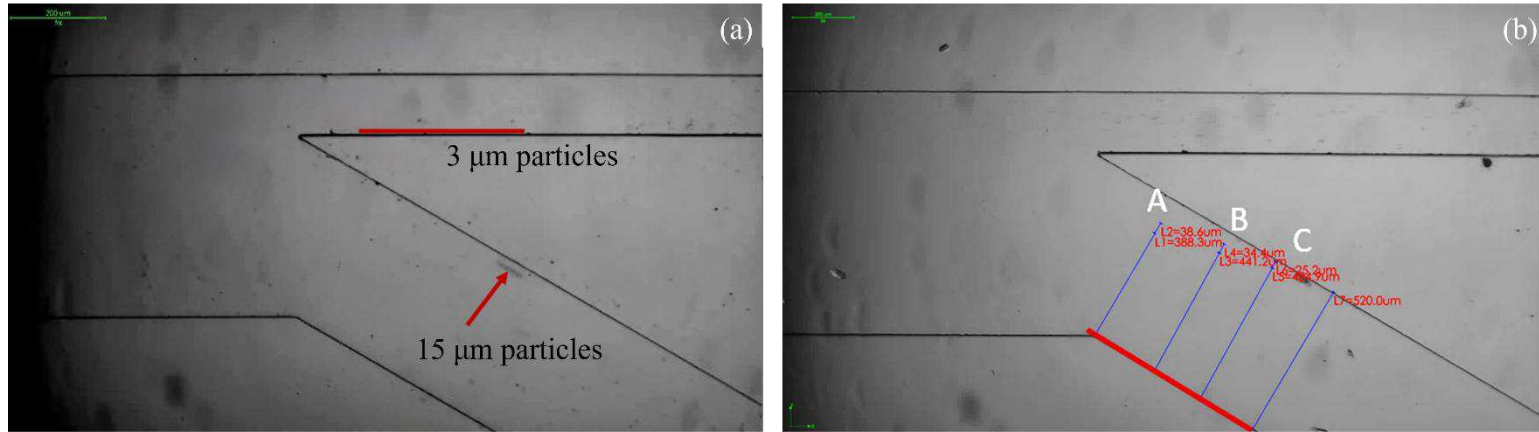


Fig. 5 Experimental separation behavior of mixed 3 and 15 μm polystyrene microparticles using the hybrid-inlet PFF device: (a) representative particle trajectories in the expanded separation region; and (b) measured particle-trajectory ranges at different locations in the separation region.

Quantitative analysis showed that the 3 μm particles exhibited a sorting rate of 90.4%, a mis-sorting rate of 7.6%, a purity of 96.2%, and a recovery rate of 98.0%. The corresponding values for the 15 μm particles were 94.4%, 4.0%, 91.8%, and 98.4%, respectively, as summarized in Table 3.

Table 3. Separation performance of mixed polystyrene microparticles using the hybrid inlet design

Particle size	Sorting Rate (%)	Mis-sorting Rate (%)	Purity (%)	Recovery Rate (%)
3 μm	90.4	7.6	96.2	98.0
15 μm	94.4	4.0	91.8	98.4

Both particle populations achieved sorting rates above 90%, demonstrating that the size-dependent displacement generated in the pinched and expanded regions was sufficient to direct most particles toward their intended outlets. The 15 μm particles exhibited a slightly higher sorting rate because their larger diameters produced a greater lateral displacement from the channel wall. Consequently, the trajectories of the larger particles were more clearly separated from those of the smaller particles after entering the expanded region.

The purity of the 3 μm fraction was higher than that of the 15 μm fraction. Only a

small number of 15 μm particles entered Outlet 1, resulting in a relatively pure small-particle fraction. In contrast, the presence of mis-sorted 3 μm particles in Outlet 2 reduced the purity of the large-particle fraction. This result indicates that sorting rate and purity represent different aspects of separation performance. Sorting rate describes the proportion of the initial target particles collected from the intended outlet, whereas purity represents the composition of the collected outlet sample.

The recovery rates of both particle sizes were approximately 98%, indicating that particle losses caused by adhesion, inlet retention, and incomplete collection were limited. The addition of 0.0001% Tween 20 reduced particle adhesion within the inlet, microchannel, outlet region, and collection tubing. In addition, shortening the tubing connected to Outlet 2 reduced hydraulic resistance and facilitated the collection of the larger particles.

To evaluate chip-to-chip reproducibility, an additional device with the same hybrid inlet configuration was fabricated and tested under identical operating conditions. The repeated experiment produced sorting rates of 90.4% and 92.5% for the 3 and 15 μm particles, respectively, with corresponding recovery rates of 98.1% and 97.8%. The similar results obtained using the two devices indicate that the hybrid inlet configuration and fabrication process provided reproducible particle-separation performance.

Although high sorting and recovery rates were obtained, complete separation was not achieved. The remaining mis-sorting may have resulted from variations in the initial particle positions, particle–wall interactions, local flow disturbances introduced by the inlet tubing, and minor deviations in the fabricated channel dimensions. In addition, the two-dimensional numerical model did not account for vertical particle positions or three-dimensional flow effects near the inlet opening, which may have contributed to the differences between the simulated and experimental results.

3.5. Separation of individual microalgal species

Following validation with standardized polystyrene microparticles, the hybrid-inlet device was evaluated using two microalgal species with distinct size ranges. *Nannochloropsis* sp. consisted mainly of approximately spherical cells with diameters of 3–5 μm , whereas *Thalassiosira weissflogii* consisted of larger cells with characteristic dimensions of approximately 11–16 μm .

Single-species experiments were first conducted to determine the appropriate operating condition for each microalgal species. *Nannochloropsis* sp. was tested at Inlet 1 and Inlet 2 flow rates of 10 and 100 $\mu\text{L}/\text{min}$, respectively, corresponding to a flow-rate ratio of 1:10. Most cells were collected from Outlet 1.

The same flow-rate ratio was subsequently evaluated using *T. weissflogii*.

However, some cells were not consistently directed toward Outlet 2 at 1:10. The flow-rate ratio was therefore reduced to 1:9 by setting the flow rates of Inlet 1 and Inlet 2 to 10 and 90 $\mu\text{L}/\text{min}$, respectively. Following this adjustment, the larger cells exhibited more stable trajectories toward Outlet 2. Representative microalgal samples and the flow-rate-ratio optimization of *T. weissflogii* are shown in Fig. 6.

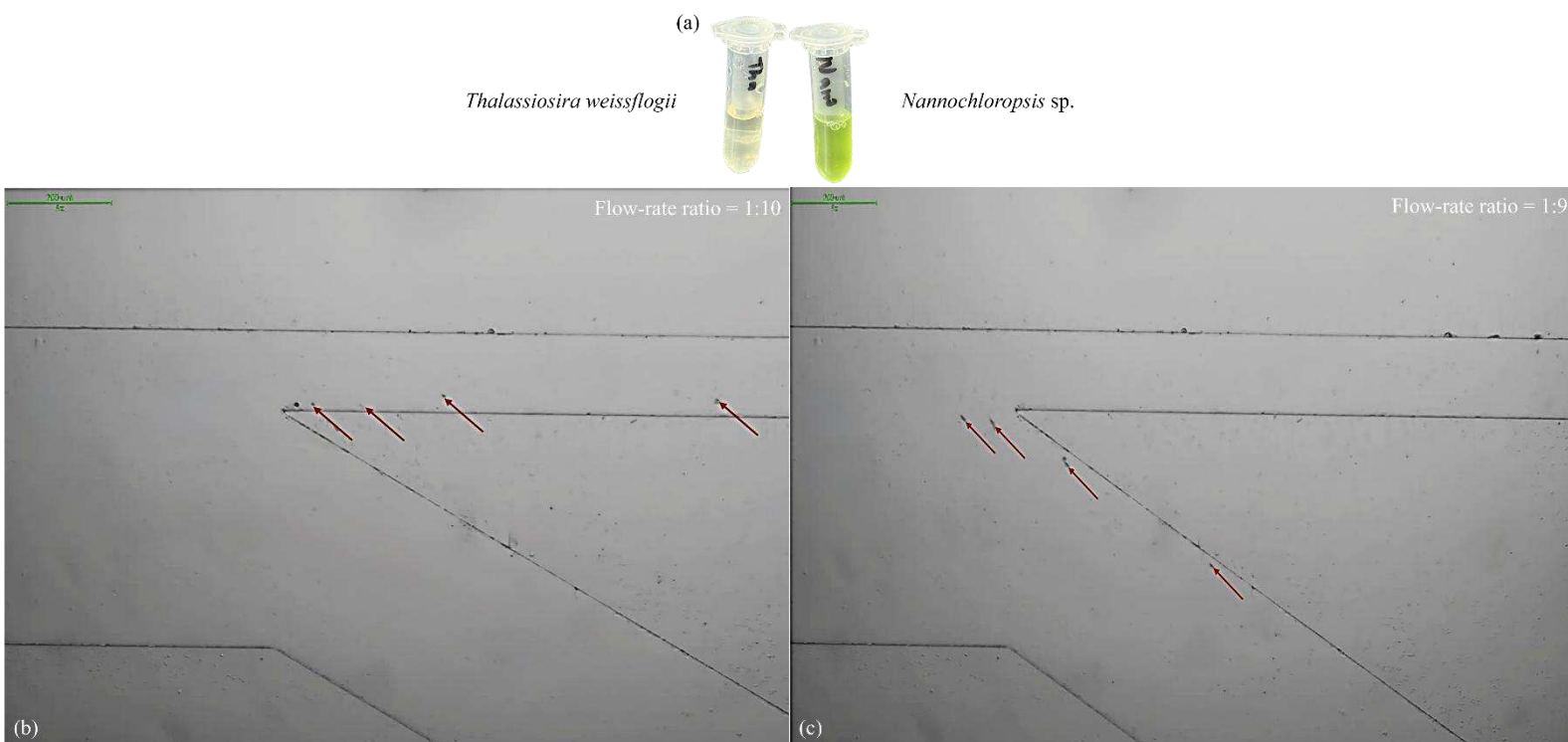


Fig. 6 Microalgal samples and flow-rate-ratio optimization: (a) samples of *Thalassiosira weissflogii* and *Nannochloropsis* sp.; and separation behavior of *T. weissflogii* at inlet flow-rate ratios of (b) 1:10 and (c) 1:9.

Quantitative analysis showed that *Nannochloropsis* sp. achieved a sorting rate of 90.7% and a recovery rate of 98.7% at a flow-rate ratio of 1:10. At the optimized flow-rate ratio of 1:9, *T. weissflogii* achieved a sorting rate of 91.0% and a recovery rate of 95.9%. The single-species separation results are summarized in Table 4.

Table 4. Separation performance of individual microalgal species

Species	Size range (μm)	Flow-rate ratio	Sorting rate (%)	Recovery rate (%)
<i>Nannochloropsis</i> sp.	3–5	1:10	90.7	98.7
<i>T. weissflogii</i>	11–16	1:9	91.0	95.9

The difference in the optimal flow-rate ratios may be related to the nonspherical morphology and broad size distribution of *T. weissflogii*. Unlike rigid polystyrene

particles with narrowly controlled diameters, biological cells may rotate, aggregate, or enter the pinched segment at different orientations. Consequently, their effective hydrodynamic sizes and trajectories cannot be predicted solely from their nominal dimensions.

These single-species experiments confirmed that both microalgal species could be directed toward their intended outlets. Because the 1:9 condition improved the collection of *T. weissflogii* while maintaining size-dependent separation, this flow-rate ratio was subsequently used for the mixed-microalgae experiments.

3.6. Separation of mixed microalgae

A mixed sample containing *Nannochloropsis* sp. and *T. weissflogii* was used to assess the practical biological separation capability of the hybrid-inlet PFF device. The separation was performed at an inlet flow-rate ratio of 1:9, corresponding to flow rates of 10 $\mu\text{L}/\text{min}$ at Inlet 1 and 90 $\mu\text{L}/\text{min}$ at Inlet 2.

A total of 1696 *Nannochloropsis* sp. cells were counted before separation. Among these cells, 1531 were correctly collected from Outlet 1, 111 were mis-sorted into Outlet 2, and 54 were unrecovered. For *T. weissflogii*, 1540 initial cells were counted, of which 1417 were correctly collected from Outlet 2, 61 were mis-sorted into Outlet 1, and 62 were unrecovered.

The resulting sorting rates of *Nannochloropsis* sp. and *T. weissflogii* were 90.3% and 92.0%, respectively. The corresponding outlet purities were 96.2% and 92.7%, while the recovery rates were 96.8% and 96.0%, respectively, as summarized in Table 5.

Table 5. Separation performance of the mixed microalgal sample

Species	Sorting Rate (%)	Mis-sorting rate (%)	Purity (%)	Recovery Rate (%)
<i>Nannochloropsis</i> sp.	90.3	6.5	96.2	96.8
<i>T. weissflogii</i>	92.0	4.0	92.7	96.0

The sorting rates above 90% demonstrated that the proposed device could distinguish the two microalgal size groups under continuous-flow operation. The purity of the *Nannochloropsis* sp. fraction was higher than that of the *T. weissflogii* fraction. This difference was primarily associated with the proportion of small cells mis-sorted into Outlet 2. Because the smaller cells occupied streamlines closer to the outlet boundary, minor variations in focusing position could cause some cells to enter the large-cell outlet. Conversely, the larger displacement of *T. weissflogii* reduced its probability of entering Outlet 1.

The recovery rates of approximately 96% were slightly lower than those obtained with the polystyrene particles. This reduction can be attributed to the biological characteristics of the microalgae. Microalgal cells possess more complex surface properties than polystyrene particles and may adhere to PDMS, glass, or tubing surfaces. In addition, *T. weissflogii* cells may exhibit irregular orientations or form small aggregates, increasing the probability of temporary retention within the inlet and channel. Nevertheless, the recovery values remained high, indicating that the surfactant treatment and modified inlet effectively limited sample loss.

The successful separation of the mixed microalgal sample is important because it demonstrates that the device performance was not limited to standardized spherical particles. Although the two selected species had clearly different size ranges, their morphology and surface characteristics were substantially more complex than those of polystyrene particles. The results therefore provide initial evidence that the proposed PFF device can be used as a label-free pretreatment platform for biological samples.

However, the present system remains primarily size-dependent. Microalgal species with overlapping dimensions may not be separated effectively using the same geometry. Future improvements could combine multiple separation stages, additional channel geometries, or optical detection to improve selectivity for species with similar sizes.

3.7. Repeatability of mixed-microalgae separation

Four independent separation experiments were conducted under the same operating conditions to evaluate the repeatability of the hybrid-inlet device. For *Nannochloropsis* sp., the sorting rate ranged from 89.7% to 90.9%, while the recovery rate ranged from 96.2% to 96.8%. The resulting mean sorting and recovery rates were $90.4 \pm 0.5\%$ and $96.5 \pm 0.3\%$, respectively.

For *T. weissflogii*, the sorting rate ranged from 90.3% to 92.6%, and the recovery rate ranged from 96.0% to 96.7%. The corresponding mean values were $91.8 \pm 1.0\%$ and $96.3 \pm 0.3\%$, respectively.

The results of the four repeated mixed-microalgae separation experiments are summarized in Table 6.

Table 6. Repeatability of mixed-microalgae separation

Run	<i>Nannochloropsis</i> sp.		<i>Thalassiosira weissflogii</i>	
	Sorting (%)	Recovery (%)	Sorting (%)	Recovery (%)
Run 1	89.7	96.2	92.6	96.7
Run 2	90.4	96.8	92.0	96.0
Run 3	90.9	96.6	90.3	96.3
Run 4	90.4	96.2	92.1	96.0
Mean $\pm$ SD	90.4 $\pm$ 0.5	96.5 $\pm$ 0.3	91.8 $\pm$ 1.0	96.3 $\pm$ 0.3

The small standard deviations indicate that the device maintained stable separation and collection performance among repeated experiments. The slightly larger variation observed in the sorting rate of *T. weissflogii* was likely caused by its broader size range and nonspherical morphology. Different cell orientations within the pinched segment may have changed the effective lateral displacement and contributed to greater trajectory variation than that of the smaller, more spherical *Nannochloropsis* sp. cells.

Despite this biological variability, all four experiments maintained sorting rates near or above 90% and recovery rates above 96%. These results demonstrate that the hybrid inlet provided reproducible sample delivery and that the selected operating condition was sufficiently stable for repeated microalgal separation.

Overall, the hybrid inlet design improved particle transport without sacrificing the hydrodynamic focusing required for PFF. The device achieved high recovery for standardized microparticles and maintained sorting rates above 90% when applied to mixed microalgal cells. The results support the use of the proposed design as a simple and passive size-based separation platform. Further validation using environmental samples, species with overlapping dimensions, and longer operation periods will be necessary to establish its applicability to more complex microalgal populations.

Conclusions

This study developed and experimentally validated a modified-inlet pinched flow fractionation microfluidic chip for the passive size-based separation of microparticles and microalgae. Numerical simulations combining laminar flow and particle tracing were used to evaluate the flow-field distribution and particle trajectories under different inlet flow-rate ratios. The simulation results showed that increasing the sheath-flow rate enhanced the hydrodynamic focusing of the sample stream and enlarged the lateral displacement between particles of different sizes in the expanded region.

Different inlet-opening configurations were investigated to address particle retention and unstable focusing near the sample inlet. The partially opened inlet provided stable particle guidance but resulted in considerable particle retention, whereas the fully opened inlet improved particle transport but caused greater variations in the initial particle position. The hybrid inlet combined the advantages of both configurations by reducing particle accumulation while maintaining stable focusing. In addition, shortening the tubing connected to Outlet 2 and adding 0.0001% Tween 20 reduced hydraulic resistance and particle adhesion, thereby improving particle collection and recovery.

The proposed device was first validated using 3 and 15 μm polystyrene microparticles and was subsequently applied to the separation of *Nannochloropsis* sp. and *Thalassiosira weissflogii*. At an inlet flow-rate ratio of 1:9, the sorting rates of *Nannochloropsis* sp. and *T. weissflogii* were 90.3% and 92.0%, respectively. Their corresponding purities were 96.2% and 92.7%, while the recovery rates were 96.8% and 96.0%. Four repeated experiments produced mean sorting rates of $90.4 \pm 0.5\%$ and $91.8 \pm 1.0\%$, together with mean recovery rates of $96.5 \pm 0.3\%$ and $96.3 \pm 0.3\%$, respectively. These results confirmed that the device maintained stable and reproducible separation performance for biological samples.

Overall, the modified-inlet PFF chip provides a simple, label-free, and reproducible approach for separating microalgae with distinct size ranges without requiring an external electric, magnetic, or acoustic field. Although the present device primarily relies on particle size and may have limited selectivity for species with overlapping dimensions, it has potential for use in microalgal sample pretreatment and other biological particle-separation applications. Further studies may focus on increasing throughput, evaluating long-term operational stability, and applying the device to more complex environmental samples.

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