Characterisation of a microfluidic hydro-trap to study the effect of straining flow on waterborne microorganisms

by

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Declaration of Authorship

I, Farzan Akbaridoust, declare that this thesis titled, 'Characterisation of a microfluidic hydro-trap to study the effect of straining flow on waterborne microorganisms' and the work presented in it are my own. I confirm that:

- This work was done while in candidature for a research degree at this University.

- The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

- Where I have consulted the published work of others, this is always clearly attributed.

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- I have acknowledged all main sources of help.

Signed: 

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Date: 

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We report a systematic study on the effect of straining flow on the harmful filamentous cyanobacterium *Anabaena circinalis* using a cross-slot type microfluidic device equipped with an advanced image-based real-time control system and simultaneously quantified by micron-resolution particle image velocimetry (micro-PIV). This enables us to monitor potential morphological damage to, and simultaneously compute the forces on, filaments in real-time while the filaments of the cyanobacterium are exposed to high strain rates of up to $42 \text{ s}^{-1}$.

Human survival is dependent on having access to clean water. The increasing occurrence of harmful cyanobacterial blooms in waterways restricts access to surface drinking water supplies by producing an unpalatable odour and taste, and even powerful toxins, endangering both humans and livestock. The management and characterisation of harmful algal blooms are most challenging as several factors play profound roles in the bloom-formation response of each microalgal group. One of the least understood, but fundamental processes in the prediction of bloom-occurrence and growth rate of cyanobacteria is the effect of fluid flow on these deleterious microorganisms.

The existence of only anecdotal evidence and sometimes opposite trends regarding the potential mechanical damage to cyanobacteria which may be caused by fluid flow, motivated us to systematically study the effect of fluid flow on *A. circinalis*. 
Previous studies relied on surrogate estimates of mean quantities of flow. Furthermore, previous conclusions regarding the mechanical damage to the filaments of cyanobacteria were based on consideration of the morphology of sub-samples from high throughput microorganisms before and after experiencing long-term turbulence. Therefore, no direct observation has been carried out to monitor single filaments of cyanobacteria, to observe and judge the occurrence of mechanical damage.

Hence, we systematically exposed the cyanobacteria to fully spatio-temporally quantified laminar flows and directly monitor each filament under straining flows. This was carried out using simultaneous micro-PIV measurements in a microfluidic hydro-trap, thereby observing the filaments exposed to high strain rates and calculating the forces exerted on them.

The results reveal that the mucilaginous sheath encapsulating the *A. circinalis* filaments may protect them from mechanical damage, a factor which was not taken into consideration in previous relevant studies. Moreover, our novel experiments open up new vistas of characterising mechanical properties of micron-sized biological materials.
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<td>ANFF</td>
<td>Australian National Fabrication Facility</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer-Aided Design</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-Coupled Device</td>
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<td>CMOS</td>
<td>Complementary Metal-Oxide Semiconductor</td>
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<tr>
<td>CDI</td>
<td>Central Difference Interrogation</td>
</tr>
<tr>
<td>CSIRO</td>
<td>Commonwealth Scientific and Industrial Research Organisation</td>
</tr>
<tr>
<td>CPU</td>
<td>Central Processing Unit</td>
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<td>DI</td>
<td>DeIonized</td>
</tr>
<tr>
<td>DSLR</td>
<td>Digital Single-Lens Reflex</td>
</tr>
<tr>
<td>ELWD</td>
<td>Extra Long Working Distance</td>
</tr>
<tr>
<td>FOV</td>
<td>Field Of View</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier Transform</td>
</tr>
<tr>
<td>ID</td>
<td>Inner Diameter</td>
</tr>
<tr>
<td>IPA</td>
<td>IsoPropyl Alcohol</td>
</tr>
<tr>
<td>LID</td>
<td>Low Image Density</td>
</tr>
<tr>
<td>MCN</td>
<td>Melbourne Centre of Nanofabrication</td>
</tr>
<tr>
<td>micro-PIV (μ-PIV)</td>
<td>micron-resolution Particle Image Velocimetry</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>NCC</td>
<td>Normalised Cross-Correlation Coefficient</td>
</tr>
<tr>
<td>PDMS</td>
<td>PolyDeMethylSiloxine</td>
</tr>
<tr>
<td>PGMEA</td>
<td>Propylene Glycol Methyl Ether Acetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PWM</td>
<td>Pulse-Width Modulation</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>Neodymium-doped Yttrium Aluminium Garnet</td>
</tr>
<tr>
<td>OD</td>
<td>Outer Diameter</td>
</tr>
<tr>
<td>PFA</td>
<td>PerFluoroAlkox</td>
</tr>
<tr>
<td>PIV</td>
<td>Particle Image Velocimetry</td>
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<tr>
<td>PDF</td>
<td>Probability Density Function</td>
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<tr>
<td>PTV</td>
<td>Particle Tracking Velocimetry</td>
</tr>
<tr>
<td>ROI</td>
<td>Region Of Interest</td>
</tr>
<tr>
<td>PoE</td>
<td>Power over Ethernet</td>
</tr>
<tr>
<td>SLWD</td>
<td>Super Long Working Distance</td>
</tr>
<tr>
<td>UV</td>
<td>UltraViolet</td>
</tr>
</tbody>
</table>
Symbols

Roman symbols

\(a\)  ellipsoid semi-principle axis in \(x\)-direction
\(b\)  ellipsoid semi-principle axis in \(y\)-direction
\(c\)  ellipsoid semi-principle axis in \(z\)-direction
\(D\)  diffusion coefficient
\(D_0\)  peak detectability
\(D_h\)  hydraulic diameter
\(d\)  diameter of four-roll mill rollers
\(d_e\)  effective particle diameter image
\(d_p\)  particle diameters
\(dA_e\)  infinitesimal surface area of ellipsoid
\(dA_i\)  projected infinitesimal surface area, where \(i = 1, 2, 3\)
\(dF_i\)  projected infinitesimal force component, where \(i = 1, 2, 3\)
\(E_i\)  elasticity modulus, where \(i = 1, 2, 3\)
\(e_{tt}\)  distance between trap centre and target distance in \(y\)-direction
\(F_i\)  force component, where \(i = 1, 2, 3\)
\(F_n\)  force (Brownian noise source)
\(f\)  frequency
\(f_{4rm}\)  frequency of the rollers of four-roll mills
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$f_c$</td>
<td>corner frequency</td>
</tr>
<tr>
<td>$f_l$</td>
<td>focal length</td>
</tr>
<tr>
<td>$f_{st}$</td>
<td>frequency of stagnation point vibration</td>
</tr>
<tr>
<td>$f_{sys}$</td>
<td>control system frequency</td>
</tr>
<tr>
<td>$f_\gamma$</td>
<td>frequency of strain rate fluctuation</td>
</tr>
<tr>
<td>$G$</td>
<td>shear modulus</td>
</tr>
<tr>
<td>$H$</td>
<td>height of four-roll mill container</td>
</tr>
<tr>
<td>$h$</td>
<td>channel height</td>
</tr>
<tr>
<td>$h_v$</td>
<td>valve height</td>
</tr>
<tr>
<td>$I_A$</td>
<td>intensity from first frame of image pair</td>
</tr>
<tr>
<td>$I_B$</td>
<td>intensity from second frame of image pair</td>
</tr>
<tr>
<td>$K_B$</td>
<td>Boltzmann’s constant</td>
</tr>
<tr>
<td>$K_C$</td>
<td>pressure to distance conversion factor</td>
</tr>
<tr>
<td>$K_P$</td>
<td>proportional gain coefficient</td>
</tr>
<tr>
<td>$L$</td>
<td>outlet channel length</td>
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<tr>
<td>$l_c$</td>
<td>fixed constriction length</td>
</tr>
<tr>
<td>$l_v$</td>
<td>variable/valve constriction length</td>
</tr>
<tr>
<td>$M$</td>
<td>magnification</td>
</tr>
<tr>
<td>$N$</td>
<td>number of realisations</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>$N_H$</td>
<td>number of pixel in image in horizontal direction</td>
</tr>
<tr>
<td>$N_W$</td>
<td>number of pixel in image in vertical direction</td>
</tr>
<tr>
<td>$n_0$</td>
<td>refractive index</td>
</tr>
<tr>
<td>$P$</td>
<td>pressure</td>
</tr>
<tr>
<td>$Q$</td>
<td>total volumetric flow rate</td>
</tr>
<tr>
<td>$R$</td>
<td>flow resistance</td>
</tr>
<tr>
<td>$Re_{D_h}$</td>
<td>hydraulic diameter-based Reynolds number</td>
</tr>
<tr>
<td>$Re_\gamma$</td>
<td>strain rate-based Reynolds number</td>
</tr>
</tbody>
</table>
Symbols

Cross-correlation coefficient map

Absolute temperature

Time

Velocity vector

Velocity component in x-direction

Far field/undisturbed velocity component in x-direction

Velocity component in y-direction

Far field/undisturbed velocity component in y-direction

Channel width

Velocity component in z-direction

Far field/undisturbed velocity component in z-direction

Fixed constriction width

Stagnation point position in x-direction

Cartesian coordinate - compressional axis

Stagnation point position in y-direction

Trap centre position in y-direction

Target position in y-direction

Cartesian coordinate - extensional axis

Initial position of target in y-direction

Greek symbols

Channel aspect ratio

First, second and third Jacobi elliptic integral transforms

\( \alpha, \alpha', \alpha'' \) at \( \lambda = 0 \)

Around \( a \) in confocal ellipsoidal coordinates

First, second and third Jacobi elliptic integral transforms

\( \beta, \beta', \beta'' \) at \( \lambda = 0 \)
Symbols

around $b$ in confocal ellipsoidal coordinates

$\Gamma, \Gamma', \Gamma''$ first, second and third Jacobi elliptic integral transforms around $c$ in confocal ellipsoidal coordinates

$\Gamma_0, \Gamma'_0, \Gamma''_0$ $\Gamma, \Gamma', \Gamma''$ at $\lambda = 0$

$\Gamma_v$ vortices of circulation

$\gamma$ strain rate

$\gamma_{eq}$ equivalent strain rate of ideal hyperbolic flow

$\gamma_{est}$ estimated strain rate

$\Delta$ auxiliary variable in confocal ellipsoidal coordinates

$\Delta_{4\, rm}$ gap between four-roll mill rollers

$\Delta P$ pressure difference

$\Delta t$ laser pulses time interval

$\Delta t_{delay}$ control system delay time

$\Delta x$ local displacement vector

$\delta$ gap between rollers and container in four roll mills

$\delta x$ local displacement in $x$-direction

$\delta x_{uncertainty}$ PIV measurement uncertainty

$\delta y$ local displacement in $y$-direction

$\delta z$ depth of field

$\delta z_c$ depth of correlation

$\epsilon$ turbulence dissipation rate per unit mass

$\epsilon_{th}$ depth of correlation threshold parameter

$\epsilon_{ij}$ strain components, where $i, j = 1, 2, 3$ and repeated indices do not denote a summation

$\epsilon_B$ Brownian motion relative error

$\zeta$ hydrodynamic drag coefficient

$\lambda$ ellipsoidal coordinate for confocal ellipsoids

$\lambda_0$ light wavelength
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \theta )</td>
<td>angle</td>
</tr>
<tr>
<td>( \kappa )</td>
<td>proportional coefficient in four-roll mills</td>
</tr>
<tr>
<td>( \kappa_t )</td>
<td>trap stiffness</td>
</tr>
<tr>
<td>( \mu )</td>
<td>dynamic viscosity</td>
</tr>
<tr>
<td>( \nu )</td>
<td>kinematic viscosity</td>
</tr>
<tr>
<td>( \nu_{ij} )</td>
<td>Poisson’s ratio, where ( i, j = 1, 2, 3 ), and repeated indices do not denote a summation</td>
</tr>
<tr>
<td>( \Upsilon )</td>
<td>auxiliary variable in confocal ellipsoidal coordinates</td>
</tr>
<tr>
<td>( \phi )</td>
<td>potential function</td>
</tr>
<tr>
<td>( \sigma )</td>
<td>standard deviation</td>
</tr>
<tr>
<td>( \sigma_{ij} )</td>
<td>stress components, where ( i, j = 1, 2, 3 ), and repeated indices</td>
</tr>
<tr>
<td>( \tau )</td>
<td>shear stress</td>
</tr>
<tr>
<td>( \Omega )</td>
<td>angular speed of the rollers of four-roll mills</td>
</tr>
<tr>
<td>( \omega )</td>
<td>rotation constant in linear velocity field</td>
</tr>
</tbody>
</table>

**Superscripts**

- \( * \) non-dimensionalised/normalised parameter
- \( - \) mean quantity
- \( \sim \) Fourier transform
- \( . \) first derivative of the position vector with respect to time

**subscripts**

- \( c \) channel with a fixed width constriction
- \( ctr \) constricted section of channel
- \( i \) Einstein’s notation
- \( j \) Einstein’s notation
- \( n \) channel with no constrictions
Symbols

\textit{tc} \quad \text{trap centre}

\textit{tp} \quad \text{target position}

\textit{v} \quad \text{channel with on-chip membrane valve}

\textit{val} \quad \text{on-chip membrane valve}
To my family and friends for their love and support.
Chapter 1

Introduction

Available water is critical to sustaining life. The occurrence of harmful cyanobacterial blooms in waterways restricts access to surface potable water supplies by producing an unpalatable taste and odour, and even powerful toxins, endangering both humans and livestock. Cyanobacteria, also known as blue-green algae, are a group of microorganisms, which are known to be the most primitive microorganisms on our planet, having existed for about three billion years.

One of the fundamental, but least understood processes in the characterisation of bloom-occurrence and growth rate of waterborne microalgae is the effect of fluid flow and in particular the small-scale structures of turbulence. Mass transfer of nutrients to microalgae cells is increased by turbulence, which consequently enhances the growth rate of the microorganisms. However, excessive levels of turbulence can inhibit the microorganisms’ growth rate. It is deduced that the turbulence dissipation rate, and the resulting strain rate are the most prominent factors in the microorganisms survival when it comes to the effect of small-scale turbulence on waterborne microorganisms (Thomas and Gibson, 1990). It is widely believed that the factors that may inhibit the growth rate are disruption of colonies (for colony-forming algae) and mechanical damage to, or destruction of, filaments (for filamentous algae). A small number of investigators have studied the effect
of small-scale turbulence on cyanobacteria and specifically the cyanobacterium *Anabaena*, reporting anecdotal evidence of suspected mechanical damage to this genus. *Anabaena circinalis*, recently renamed *Dolichospermum circinale* (Wacklin et al., 2009), is a filamentous, bloom-forming, cyanotoxin-producer species of cyanobacteria, which create significant water quality problems worldwide, particularly in Australia.

To investigate the effect of small scale turbulence on waterborne microorganisms, some researchers used turbulence generator fatalities such as oscillating grid tanks (Hondzo and Lyn, 1999), Taylor-Couette chambers (Mitsuhashi et al., 1995), stirring devices (Xiao et al., 2016), to name but a few. However, Micron-sized organisms are usually much smaller than the Kolmogorov length scale that represents the smallest size of eddies in a turbulent flow, which is about a few millimetres for natural environments (Sullivan et al., 2003). Therefore, it is deducted that microorganisms are exposed to a laminar flow (Whitton and Potts, 2000). This fact enables researchers to use laminar flows to investigate the effect of strain rate on waterborne microorganisms. Therefore, some researchers used laminar flows generated in Taylor-Couette facilities to study the effect of flow on microalgae (Moisander et al., 2002; Warnaars and Hondzo, 2006).

The existence of anecdotal evidence and sometimes reports of opposite trends in the literature is rooted in a few points, summarised below:

- The sensitivity to turbulence and tolerance of strain rate and the resulting forces are strongly genera and species related. However, some results were reported only based on the genera of cyanobacteria (Moisander et al., 2002).

- Previous studies relied on surrogate estimates of mean quantities of turbulence and they do not consider the spatio-temporally resolved intermittent structure of small-scale turbulence (Hondzo and Wuest, 2008). Therefore, any potential mechanical damage may be associated with sudden and localised events.
Some of the facilities such as Taylor-Couette chambers may lead to biased results due to the damage caused the microorganisms striking the wall of the device or collisions of the cultures (Warnaars and Hondzo, 2006).

Although some researchers generated laminar flows in Taylor-Couette facilities, due to the relatively large size of the agglomeration of the cultures (compared to the gap between the cylinders), these flows are not identically the same as the flow with the absence of the cultures. Thus there is still a possibility of the collision of the cultures to the solid surface of the device. In addition, laminar flows in Taylor-Couette facilities lack a spatially uniform strain rate (Moisander et al., 2002).

Conclusions regarding the mechanical damage to the filaments were based on consideration of the morphology or viability of the sub-samples from high throughput microorganisms before and after experiencing long-term turbulence. No direct observation has been carried out to monitor the single filaments of cyanobacteria, to observe and judge the occurrence of mechanical damage (Mitsuhashi et al., 1995).

In this study, in order to observe the potential damage to *Anabaena circinalis* filaments that may be caused by the strain rate of the flow, we use laminar flows with spatio-temporally uniform strain rates. To establish a spatio-temporally strain rate flow we use a cross-slot type microfluidic device equipped with an advanced real-time control system to immobilise single micron-sized target objects in the flow. The flow in the cross-slot type microfluidic device consists of two laminar streams converging at the cross-junction perpendicular to two laminar streams diverging from the cross-junction. A pure extensional stagnation point flow with spatially uniform strain rate is created in the junction-slot junction of the microfluidic device. Using an image-based control technique and implementing an on-chip membrane valve on one of the outlets (Tanyeri et al., 2010), the flow in the microchannel is actively controlled, thereby enabling the confinement of single
objects at the stagnation point of the flow. This enables us to monitor single filaments of *Anabaena circinalis* under a uniform strain rate in stagnation point flows in real-time.

Prior to exposing *A. circinalis* to the straining flow, we systematically measure the velocity fields and compute the strain rates at different flow rates using micron-resolution Particle Image Velocimetry (micro-PIV). We characterise the flow to identify the uniform strain rate region with the absence of the *A. circinalis* filaments. Moreover, we later measure the velocity field around the filaments of cyanobacteria while they are trapped in the stagnation point. This enables us to calculate the stretching and compressing forces on a target trapped in the microfluidic device. Moreover, it opens new vistas of characterising mechanical properties of micron-sized objects. No such measurements were carried out when a micron-size object was confined at the stagnation point and all previous studies were based on the measurement of the ideal flow with the absence of the trapped object.

Therefore, the aims of this study are categorised into two points:

- From a microbiological point of view we intend to observe potential mechanical damage to *A. circinalis* filaments that may be caused by the strain rate of flow.

- From a fluid mechanics point of view we intend to improve the applicability and capability of single object trapping in the straining flow by integrating this technique with simultaneous micro-PIV measurements.

All the experiments in this thesis (except the fabrication of the microfluidic devices) were conducted by the author at the Walter Bassett Aerodynamic Laboratory (located at the University of Melbourne) and all of the fabrication steps performed by the author at the Melbourne Centre of Nanofabrication (MCN).
1.1 Thesis outline

A broad outline of this thesis is as follows:

Chapter 2 will review experimental methods used in previous works that investigated the effects of fluid flow on waterborne microorganisms and the results reporting the mechanical damage to the microalgae. Based on this, we propose our approach and experimental method (the cross-slot type microfluidic trap) in order to observe and quantify the potential damage to *A. circinalis*. Moreover, the literature relevant to the cross-slot type microfluidic trap is reviewed and a modification to improve this microfluidic device is proposed.

Chapter 3 provides the information about fractionation of the cross-slot type microfluidic device using soft-lithography. The fabrication protocol of a double-layer microfluidic device (a cross-slot microchannel with an on-chip membrane valve) is detailed.

Chapter 4 describes the experiments in the cross-slot type microfluidic device in which micro-PIV measurements are used to measure the velocity field and calculate the strain rate. The aims of these experiments are to calibrate the strain rate versus flow rate and also to determine the spatially uniform strain rate region in the cross-slot junction. The results including the velocity field, strain rate and the steadiness of the flow are compared with the similar laminar flow generated in a miniature Taylor’s four-roll mill reported in appendix A.

Chapter 5 reports the use of a microfluidic trap to confine single filaments of *A. circinalis*. The implementation of the microfluidic trap, which is equipping the cross-slot microchannel with a real-time feedback control is thoroughly explained. A large number of *A. circinalis* filaments are confined in the microchannel and are imaged while exposed to the characterised and controlled straining flows.

Chapter 6 presents a novel experiment in the cross-slot microfluidic trap in which micro-PIV measurements and real-time control are simultaneously carried out in
order to measure the velocity field around a trapped target (an *A. circinalis* filament). This experiment enables us to compute the flow stresses and the exerting forces on a trapped target and also visualise the flow around it.

Lastly, the final conclusions of the study and possible directions for the future works are presented in chapter 7.
Chapter 2

Literature review and motivation

By expressing the adverse influence of harmful cyanobacteria on the environment, the significance of a cyanobacteria-associated investigation will be illustrated in this chapter. Available papers that study the effects of fluid flow on these waterborne microorganisms will be reviewed. Critically, we assess their experimental methods from a fluid mechanics view-point, and focus on the results of the mechanical damage to the cyanobacterium *Anabaena*. Based on this, we will propose our approach (that was not previously used in this field) in order to observe and quantify the potential damage to *Anabaena*. Next, the literature related to our method (in other fields) will be reviewed and a modification to improve the method will be proposed. Finally, we will clarify the research question and objectives of this thesis from both a microbiological and a fluid mechanics point of view.

2.1 A brief introduction to cyanobacteria

Water is critical for life. The increasing occurrence of harmful cyanobacterial/algal blooms in waterways restricts access to surface drinking water supplies by producing an unpalatable odour and taste, and even powerful toxins, endangering both humans and livestock (Falconer, 2005).
Cyanobacteria are a phylogenetically coherent group of organisms, which are known to be the most primitive and successful microorganisms on our planet, having existed for about three billion years. Due to their similar behaviour and appearance to algae, they are also known as blue-green algae, as they convert sunlight to biomass using photosynthesis and also have pigments that impart the cyan (blue-green) colour to them. This ancient class of photosynthetic microorganisms is the first type of organisms that released elemental oxygen into the primitive atmosphere of the Earth about two billion years ago. Cyanobacteria, considered the ancestors of all plants today, are naturally widespread in aquatic systems, equivalent to trees and grasses on land. Not all the species of cyanobacteria are harmful or toxic. Some genera of cyanobacteria are notorious for impairing drinking water by giving it an undesirable musty and earthy taste and unpleasant odour, whilst some are capable of producing the most powerful toxins known, endangering the lives of humans and animals. These adverse effects of harmful cyanobacteria on drinking water sources have led to the death of large numbers of animals, significant human illnesses and the abandonment of many recreational water resources worldwide, particularly in Australia (Chorus and Bartram, 1999; Codd et al., 2005; Falconer, 2005; Flores and Herrero, 2010; Hudnell, 2010; Trevino-Garrison et al., 2015; Westwood, 2003). The increasing formation of toxic cyanobacterial blooms is caused by the provision of their growth conditions such as warm and calm, nutrient-rich and well-illuminated water. Human activities such as draining fertiliser-rich agricultural water and sewage into rivers and the increase in the global temperature due to modern agricultural methods are also responsible for providing the favourable conditions for triggering algal bloom occurrence. Moreover, in some regions of the biosphere such as Australia the natural climatic conditions, construction of large dams and regulation of the rivers promotes the recurrence of harmful algal blooms (Brookes et al., 2002; Steffensen, 2001).
2.2 Human and animal-associated morbidities and mortalities caused by cyanobacteria

In accordance with Codd et al. (2005), based on the then-available knowledge, the first incident report of the adverse effect of harmful cyanobacteria dates back to about two centuries ago, when the death of cattle and fish were reported in Denmark by Hald (1833). That incident was followed by the publication from Francis (1878) - the pioneer of South Australian water quality research (Codd et al., 2015) - reporting the deaths of sheep, cattle, horses, pigs and dogs due to drinking water intoxicated by the cyanobacterium, *Nodularia spumigena*, in Lake Alexandrina, South Australia. A few years later, another incident involving the deaths of farm animals, ducks, chickens, pigeons and fish was reported in West Prussia (Poland) due to the existence of the cyanobacteria *Microcystis* and *Anabaena* in Lake Barlewice (Benecke, 1884). These incidences were followed by reports of illness and skin irritation in the local people who had been in contact with the cyanobacterial scum of the rivers mentioned (Codd, 1995). Since then, due to the increasing occurrence of cyanobacterial blooms, a large number of reports and articles indicates increasing animal mortalities and human morbidities due to drinking and contact with water containing cyanobacteria.

Hayman (1991) determined that the “Barcoo fever”, once known as an infectious disease during its outbreak more than a century ago, was instead caused by toxicity from cyanobacteria. Early reports of skin irritation and positive intradermal skin tests amongst the swimmers who were exposed to the water intoxicated with cyanobacteria were listed in an article by Schwimmer (1968). Negri et al. (1995) reported the first sheep mortalities caused by drinking water containing a *Anabaena circinalis* bloom. A large number of examples of animal deaths that happened before 1990 can be found in Schwimmer (1968) and Yoo (1995). More recent episodes of dog deaths in Scotland, Ireland and New Zealand due to drinking water intoxicated by anatoxin-a produced by cyanobacteria were reported by Edwards et al.
(1992); James et al. (1997); Hamill (2001), respectively. Also human-associated illnesses caused by contact with cyanobacteria are documented by Carmichael (1997, 2001); Duy et al. (2000).

The increase in the formation of harmful algal blooms is not limited to Australia and Europe. Eleven outbreaks of toxic cyanobacterial bloom and related illnesses in 2009 and 2010 were reported in the United States, whereas only three outbreaks of algal related diseases were reported there, from the year 1978 to 2008 (Hilborn et al., 2014; Trevino-Garrison et al., 2015).

Amongst all the cyanobacterial bloom incidents and related outbreaks reported, perhaps the most catastrophic occurred in 1991 in South Australia. A massive cyanobacterial bloom dominated by *Anabaena circinalis* extended over 1000 km of the third longest Australian river, the Darling River, which led to an estimated 10,000 livestock deaths in New South Wales, drawing national attention and requiring emergency water supplies for several nearby towns (NSW Blue-Green Algae Task Force (1992); Al-Tebrineh et al. 2012; Bowling and Baker 1996). A few years later, another incident that is believed to have been the cause of waterfowl deaths occurred in the city of Adelaide. Torrens Lake, once a potable water supply, experienced an outbreak of the cyanobacterium *Microcystis aeruginosa* for three successive summers (Steffensen, 2001).

## 2.3 Prevalent cyanobacteria species in Australia

Amongst the cyanobacteria species, *Microcystis aeruginosa*, *Anabaena circinalis* and *Cylindrospermopsis raciborskii* are of significant importance in Australia, with typical images of each shown in figure 2.1. They are the three most prevalent cyanotoxins-producers and bloom-forming species in Australia, which create major freshwater quality problems (Falconer, 1999, 2001).
Chapter 2. Literature review and motivation

2.3.1 Anabaena circinalis (Dolichospermum circinale)

Anabaena circinalis Rabenhorst, (recently renamed Dolichospermum circinale Rabenhorst by Wacklin et al. 2009) is capable of producing various type of toxins such as saxitoxins, anatoxins and microcystins. However, the type of toxins produced is region-specific. For example, saxitoxins (Paralytic Shellfish Poisons) occur amongst the Australian blooms of A. circinalis. This class of neurotoxin is one of the most potent naturally produced toxins known. Ingestion of a sufficiently high dose of saxitoxins leads to respiratory paralysis and eventually death in both animals and humans, due to the disruption of the signals between muscles and nerves.
(Burch, 2001; Humpage et al., 1994). For more recent information regarding toxicity of different species of cyanobacteria the reader is directed to the “Handbook of Cyanobacteria” by Sarma (2012).

2.3.1.1 Cellular structure of *Anabaena circinalis*

*Anabaena* is a morphologically filamentous and usually heterocystous cyanobacterium. Filaments of *Anabaena* and some other filamentous cyanobacteria consist of (a few to hundreds of) connected cells, which are mainly vegetative cells that are responsible for photosynthesis. When they are deprived of nitrogen compounds such as nitrate and ammonia, they develop another type of cell, heterocyst, which functions to fix nitrogen (converting the environmental nitrogen (N$_2$) to the nitrogen compounds). Heterocysts are usually round and lighter in color and larger in size than the vegetative cells (Flores and Herrero, 2010). Another type of cell that can be developed in the filamentous cyanobacteria is the akinete, which assists the microorganisms survival while exposed to cold (but not heat) and desiccation conditions. The fourth type of cell is a hormogonium, which is formed as a separate motile filament that can serve a dispersal function. Generally, development of the last two types of cell mentioned is rarer than the others. Figure 2.2 depicts the compartments of vegetative and heterocyst cells and their connection in a filament of *Anabaena*. Comprehensive information regarding the cell types and functions, their connections and their molecular exchange is given by Flores and Herrero (2010).

Apart from developing heterocysts in a low nitrogen compound medium and developing akinetes in low temperature and desiccation conditions, producing a protective mucilaginous covering around the filaments is a defence mechanism that cyanobacteria have evolved to protect themselves from predators and grazers such as ciliate grazers. Production of a mucilaginous sheath is a physical defence method that assists the microalgae in defending themselves from deleterious
Figure 2.2: Compartments of vegetative and heterocyst cells and their connection in a filament of 
*Anabaena* (Flores and Herrero, 2010).
Chapter 2. Literature review and motivation

Figure 2.3: A filament of *Anabaena* encapsulated with the translucent mucilaginous sheath ([www.biologie.uni-hamburg.de](http://www.biologie.uni-hamburg.de)) by Prof. Celia Smith, University of Hawai’i at Mānoa.

Microorganisms by disruption of the grazer colonization or growth of parasites. However, the production of the sheath does not necessarily confirm the existence of the deleterious microorganisms ([Amsler, 2008; Pajdak-Stós et al., 2001]). Figure 2.3 shows a filament of *Anabaena* encapsulated with a mucilaginous sheath that is translucent and barely visible through bright-field microscopy.

In this research we focus on *A. circinalis* from two Australian rivers, the Canning River and the Murrumbidgee River (details of isolation and maintenance will be provided in section 5.6).

The Department of Health of the Government of Western Australia has been warning the members of public to avoid recreational activities in the Canning River, due to an outbreak of *A. circinalis* and formation of a bloom in this river persisting since 2016. This outbreak is still threatening pets, livestock and human health by causing illnesses such as gastro-intestinal diseases, which are caused by influencing nerve tissue and consequently leading to liver damage. Skin irritation and dermatitis in humans also may be caused by contact with this harmful bloom. Moreover, bioaccumulation of one of the toxins produced by *A. circinalis* in this river in shellfish may lead to paralytic shellfish poisoning, and respiratory arrest in extreme cases.

*A. circinalis* in the Murrumbidgee River, New South Wales, Australia is responsible for a disagreeable metabolically-produced taste and odour by producing
geosmin in the Murrumbidgee River and may produce toxins as well. However, generally being odorous does not necessarily confirm the existence of toxins in blooms (Bowmer et al., 1992).

2.4 Strategies to characterise harmful cyanobacterial blooms

As discussed above an overwhelming number of incidents due to the outbreak of cyanobacterial blooms have occurred throughout the last century, causing a large number of animal mortalities and human morbidities. Different strategies have been taken into account to prevent the formation of harmful algal blooms and the removal of the toxins from the blooms (Codd et al., 2005; Qian, 2012). The first step to implementing barriers against harmful bloom occurrence is to identify responses of cyanobacteria to different environmental conditions.

The management and characterisation of algal blooms are most challenging as several factors play profound roles in the bloom-formation response of each microalgal group. Temperature, exposure to nutrients, light conditions and flow conditions such as flow rates and stratification are some of the factors that can be considered for predicting the behaviour of bloom-forming micro-algae (Cook et al., 2010).

2.5 The effects of fluid flow on phytoplankton with an emphasis on mechanical damage to cyanobacteria

One of the least understood, but fundamental processes in the prediction and characterisation of bloom-occurrence and growth rate of cyanobacteria and other
waterborne microalgae is the effect of fluid flow and in particular the small-scale structures of turbulent flows (Fogg et al., 1960; Hondzo and Lyn, 1999; Hondzo and Wuest, 2008; Michels et al., 2010; Mitsuhashi et al., 1995; Moisander et al., 2002; Thomas and Gibson, 1990; Wilkinson et al., 2016; Xiao et al., 2016).

Growth rates of *Anabaena* and other cyanobacteria under different conditions have been reported by many researchers (e.g. Howarth et al. (1993); Westwood and Ganf (2004) and many more articles). Whereas, a relatively smaller number of investigators have studied the effect of small-scale turbulent flows on cyanobacteria and specifically *Anabaena*, reporting anecdotal evidence of suspected mechanical damage. Fogg et al. (1960) reported *Anabaena cylindrica*, Moisander et al. (2002), Paerl and Tucker (1995) reported *Anabaena sp.* (i.e. with no species specification) and Xiao et al. (2016) reported *Anabaena flos-aquae*. Regarding *Anabaena circinalis*, no systematic study has reported the mechanical damage to this species.

Thomas and Gibson (1990) stated that mass transfer of nutrients and solutes to microalgal cells is increased by turbulence, which consequently enhances the growth rate of the microorganisms. However, excessive levels of turbulence can inhibit or halt the microorganisms’ growth rate. They deduce that the factors that may inhibit the growth rate are disruption of colonies (for colony-forming algae) and mechanical damage to, or destruction of, filaments (for filamentous algae) and even cell lysis in some cases. They hypothesised that the sensitivity to growth inhibition by turbulence is microalgal group specific, with *green algae* < *blue-green algae* < *diatoms* < *dinoflagellates*. They and other researchers, Hondzo and Wuest (2008); Moisander et al. (2002) to name but a few, deduced the turbulence kinetic dissipation rate ($\epsilon$), and the resulting strain rate,

$$\gamma = \left(\frac{\epsilon}{\nu}\right)^{\frac{1}{2}},$$  \hspace{1cm} (2.1)

where $\nu$ is the kinematic viscosity, and the resulting shear stress,
\[ \tau = \mu \gamma, \]  

(2.2)

where \( \mu \) is the dynamic viscosity, are the most crucial factors in the microorganisms survival when it comes to the effect of small-scale turbulence on waterborne microorganisms (Thomas and Gibson, 1990).

Fogg et al. (1960) studied the growth rate of the cyanobacterium *Anabaena cylindrica* under different conditions in a reciprocal shaker. They did not characterise the turbulent flow in their apparatus, such as the turbulence dissipation rate, and their results were presented based on the degrees of shaking. The results revealed that an increase in the rate of shaking led to an increase in the growth rate, whereas by applying excessive rates of shaking, the growth rate dramatically decreased and was eventually halted. Based on their results, Thomas and Gibson (1990) concluded that mechanical damage to *A. cylindrica* filaments might be the reason for the inhibited growth.

Savidge (1981) compared the growth rate of a diatom and a green alga. At the same agitation rate the green alga showed more growth than the diatom.

Thomas and Gibson (1990) stated that Brian A. Whitton (priv. comm.) observed obvious signs of mechanical damage to filaments due to turbulence. However, to our knowledge no articles or books were written thereafter by Prof. B. A. Whitton explicitly stating the observation of mechanical damage due to turbulence.

Thomas et al. (1984a,b,c, 1986) did not observe any mechanical cell damage to a diatom, two different green algae and several marine microalage, despite exposing cultures to high levels of turbulence in various turbulence generator facilities. It was contrary to the statement by Thomas and Gibson (1990) that high levels of turbulence can physically damage microalgae.

Generally, damage to the filaments and cells of waterborne microorganisms leads to fast death or “crashes” (Paerl, 1990). Paerl and Bland (1982) noted that in
N$_2$ fixing filament, if the connection between the heterocyst and photosynthetic cells breaks, the heterocyst will not be capable of fixing N$_2$, but will senesce and eventually be shed from the filament. Paerl and Tucker (1995) proposed that *Anabaena* filaments may be physically disrupted by accruing structural damage such as filament breakage and lysis. They deduce that connections between photosynthetic heterocysts and neighbour photosynthetic/vegetative cells are prone to disruption because of their narrow and fragile appearance. However, they did not carry out any experiment to prove this statement and they referred to articles (Hunt, 1982; Paerl and Bland, 1982) that did not observe the breakage of filamentous cyanobacteria by exposure to turbulence. They also noted that filament breakage is more prevalent amongst the species existing as solitary filaments such as *Anabaena circinalis*, *A. cylindrica* and *A. oscillarioides* compared with those that have intertwined and aggregated filaments such as *Anabaena flos-aquae* and *Aphanizomenon flos-aquae*.

In addition, being filamentous assists cyanobacteria in mat-formation (united in bundles of intertwined filaments). Mat-formation makes filamentous cyanobacteria buoyant, thereby promoting floating to the better illuminated water surface region.

Mitsuhashi et al. (1995) exposed the cyanobacterium *Spirulina platensis* and the green alga *Chlorella vulgaris* to the turbulence shear stresses in a Taylor-Couette flow facility to characterise the effect of the shear flow at different temperatures and illumination conditions on photosynthetic activities of these microorganisms. Prior to their experiments they degassed the dilute medium of the cultures and they noted that the shear stresses definitely broke the filaments of the cyanobacterium. This conclusion was based on comparison of the average length of the filaments (under a microscope) before and after experiencing turbulence. They also concluded that the decrease in oxygen production was caused by mechanical damage (resulting from the increase in the shear stress) to filaments and cells. However, different trends of oxygen production can be seen in their plots for various light and temperature conditions. In contrast with their expectations they
Table 2.1: Average dissipation rates and the resulting strain rates in nature from various part of the world.

<table>
<thead>
<tr>
<th>Site</th>
<th>$\epsilon$ (m$^2$s$^{-3}$)</th>
<th>$\gamma$ (s$^{-1}$)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oceans and lakes</td>
<td>$10^{-7}$</td>
<td>0.32</td>
<td>Warnaars and Hondzo (2006)</td>
</tr>
<tr>
<td>Open oceans</td>
<td>$\leq 3.8 \times 10^{-7}$</td>
<td>0.62</td>
<td>Reynolds (2006)</td>
</tr>
<tr>
<td>A neap tide</td>
<td>$10^{-4}$-$10^{-3}$</td>
<td>0.31-0.6</td>
<td>Sharples et al. (2007)</td>
</tr>
<tr>
<td>Lake Minnetonka</td>
<td>$3 \times 10^{-6}$</td>
<td>1.73</td>
<td>Wilkinson et al. (2016)</td>
</tr>
<tr>
<td>Lake Baikal</td>
<td>$5 \times 10^{-11}$</td>
<td>0.01</td>
<td>Lorke and Wüest (2002)</td>
</tr>
<tr>
<td>Irish Sea</td>
<td>$\leq 4 \times 10^{-5}$</td>
<td>6.32</td>
<td>Reynolds (2006)</td>
</tr>
<tr>
<td>Lake Müggelsee</td>
<td>$5 \times 10^{-8}$</td>
<td>0.22</td>
<td>Lorke and Wüest (2002)</td>
</tr>
<tr>
<td>Baltic Sea</td>
<td>$5 \times 10^{-11}$</td>
<td>0.01</td>
<td>Züllicke et al. (1998)</td>
</tr>
<tr>
<td>Lake Neuchatel</td>
<td>$5 \times 10^{-9}$</td>
<td>0.03</td>
<td>Lorke and Wüest (2002)</td>
</tr>
</tbody>
</table>

Also observed a decrease in the oxygen production of the green alga, albeit having thick strong cell walls and no observation of mechanical damage to the green alga.

It should be noted that the levels of turbulence in their experiments were much higher than previous works and higher than the turbulence that is experienced by microalgae in their natural habitat. They applied a maximum averaged shear stress of 0.6 Pa, and reported that the filament fragmentation happened at shear stress greater than 0.2 Pa. These numbers (using equation 2.1) correspond to dissipation rates and strain rates of $\epsilon = 4 \times 10^{-2}$ m$^2$s$^{-3}$, $\gamma = 200$ s$^{-1}$ and $3.6 \times 10^{-1}$ m$^2$s$^{-3}$, $\gamma = 600$ s$^{-1}$, respectively.

However, the common dissipation rates in oceans and lakes is $10^{-7}$ m$^2$s$^{-3}$ (Warnaars and Hondzo, 2006) and at a neap tide it typically reaches $10^{-4}$-$10^{-3}$ m$^2$s$^{-3}$ (Sharples et al., 2007), or in accordance with other reports it generally varies between $1 \times 10^{-11}$ and $4 \times 10^{-5}$ m$^2$s$^{-3}$ in nature. For instance, dissipation rates of $3 \times 10^{-6}$ m$^2$s$^{-3}$ (Wilkinson et al., 2016) and $5 \times 10^{-8}$ m$^2$s$^{-3}$ (Lorke and Wüest, 2002) were reported for Lake Minnetonka in the United States and Lake Müggelsee in Germany, respectively. More examples of average dissipation rates and the resulting strain rates from various regions of the world are listed in table 2.1 and more can be found in Reynolds (1994, 2006). Using equations 2.1 and 2.2 and assuming $\mu = 1$ mPa.s, the average shear stresses can be calculated.
Hondzo and Lyn (1999) investigated the effect of small-scale turbulence on the green alga *Scenedesmus quadricauda*. Using an oscillating grid tank they generated isotropic turbulence with a reasonable range of dissipation rate comparable with nature. Then they exposed the green alga to small-scale turbulence. By measuring the amount of chlorophyll-a in the sub-samples periodically taken from the grid tank every 12 or 24 hours after experiencing turbulence they reported that the mechanical damage to the cells was obvious. However, cell morphometry was not consistent with different flow conditions.

Moisander et al. (2002) studied the effects of the small-scale shear stress on three different heterocystous cyanobacterial genera from the Baltic Sea. They studied the effects of shear stress on CO$_2$-fixation, nitrogenase activities, pH, dissolved inorganic carbon, and also morphology (filament length) of the cyanobacteria using laminar shearing flows in Taylor-Couette chambers. They carried out laboratory (culture) experiments on two different strains of *Nodularia* (*Nodularia sphaerocarpa* and *Nodularia spumigena*) and they also conducted shipboard experiments on *Nodularia*, *Aphanizomenon* and *Anabaena*. Their general conclusion was that CO$_2$ fixation and nitrogenase activities decreased when the shear rate increased.

From a morphological point of view, the *Anabaena* and *Aphanizomenon* filaments generally decreased in length, whereas for the *Nodularia* no identical trend can be seen in their results.

Regarding the results of their culture experiments, Moisander et al. (2002) reported that the filament length of *N. sphaerocarpa* decreased (possibly due to morphological damage) whereas those of *N. spumigena* remained unchanged. However, the standard deviation in lengths of both strains before and after experiencing shear flow were greater than the changes in filament length.

For the field experiments, they conducted the same type of experiments (Taylor-Couette) but with changes in the duration and shear rates. The experiments were carried out in the field during July and August of four successive years starting
in 1997. Different trends can be seen in their results for different years. Possibly due to the variety of the species in the samples taken from the Baltic Sea and the difficulty of isolating the strains onboard they did not isolate the species of the cyanobacterial genera. Therefore, their report was only genus-specific although they had mentioned cyanobacteria activities and responses are both genus and species specific.

Regarding their field experiment results for *Nodularia*, the length of *Nodularia* increased by twice its initial length in the year 1998, whereas it did not show any changes in the experiments performed in the years 1999-2000, in spite of experiencing lower shear in shorter duration. That was in contrast with their culture experiments, possibly due to the different initial physiological conditions of the strains and considering the results from all the strains/species of *Nodularia* together.

Regarding the *Anabaena* and *Aphanizomenon* field experiments in 1998 the length of filaments of both genera dramatically decreased, however, in the experiments in 1999-2000 with more pairs (experiments) the decreasing trend became less significant. They proposed that the possible mechanism of the decrease in the filament length was filament breakage and referred to the paper reviewed above (Mitsuhashi et al., 1995). However, they also mentioned the dissipation rate applied in Mitsuhashi et al. (1995) was orders of magnitude higher than their dissipation rate, and also higher than the common dissipation rate in nature.

They concluded that the shear stress adversely affects physiological activities such as N\textsubscript{2} and CO\textsubscript{2} fixation prior to morphological damage. They also claimed their data is consistent with a data reported in a Master’s thesis carried out in their group (unpublished). By comparing *Anabaena* and *Aphanizomenon* to *Nodularia*, they suggested that the former genera are perhaps more prone to turbulence and breakage, although as mentioned before, the senior author (H. W. Paerl) in his
previous article had mentioned that *Aphanizomenon flos-aquae* is a turbulence-tolerant species (Paerl and Tucker, 1995).

It should be noted that there was either a miscalculation in turbulence characteristics or error of the chamber dimensions in their article (Moisander et al., 2002). Substituting the dimensions of the chambers into the shear stress equation of laminar flow in the Taylor-Couette facility gives different values to what they reported. Also the dimensions of the chamber do not match the reported value for the ratio of the gap to the outer diameter of the chamber.

From their results it can be concluded that morphological damage (if it is the mechanism of decrease in filament length) strictly is a function of the initial health/physiological condition of the cyanobacteria and is strictly species-specific.

Warnaars and Hondzo (2006) investigated the growth of the green alga *Selenastrum capricornutum* in turbulent flows generated in two different facilities, a Taylor-Couette facility and a tank with two submersible sound speakers. They concluded that the latter is superior to the former by mimicking the flow in natural environments. The disadvantage of the Taylor-Couette facility was the existence of the large surface area (of the cylinders) that is in contact with the culture in comparison with the other setup. More importantly, the existence of the velocity gradient and the greater centrifugal force close to the inner cylinder propelled the cells towards the outer cylinder, thereby increasing the possibility of impacting the cells to the outer cylinder leading to uncontrolled cell damage.

Hondzo and Wuest (2008) carried out two sets of laboratory experiments in isotropic turbulent flows generated by two stand-alone setups, an oscillating grid tank and another tank with two submersible sound speakers each with a fixed grid in front. The former was used to expose a proteobacterium *Escherichia coli*, whilst the latter was used to expose a green alga, *Selenastrum capricornutum* to small scale turbulence. Their results showed that the relative growth rate (growth rate in turbulence to that in stagnant water) of *E. coli* increased five times after two
days of experiencing turbulent flow at $\epsilon = 1.4 \times 10^{-4} \text{ m}^2\text{s}^{-3}$, whereas the growth rate of *S. capricornutum* increased almost twice at $\epsilon = 3.5 \times 10^{-7} \text{ m}^2\text{s}^{-3}$ and by further increase in the turbulence intensity to $\epsilon = 1.5 \times 10^{-4} \text{ m}^2\text{s}^{-3}$ the growth rate decreased to 1.4. They deduced the decrease in viability was associated with cell damage due to the strain rate. They concluded that small-scale turbulence considerably affects the nutrient uptake and growth rate of microalgae. Finally they modified the Monod-type equation (a mathematical model for the growth of microorganisms) by integrating the turbulence effect into the equation as a constant.

Michels et al. (2010) investigated the effect of shear stress on viability of a diatom *Chaetoceros muelleri*. Similar to Mitsuhashi et al. (1995) they applied extreme shear stresses and rates to the *C. muelleri* using laminar flows in a Taylor-Couette facility. They intended to obtain a threshold value for shear stress to design a large scale production system for *C. muelleri*. The results (using fluorescent staining methods) showed that the viability of *C. muelleri* decreased at very high shear stresses and also the effect of shear stress was almost spontaneous. Although the viability of the *C. muelleri* decreased by the increase in the shear, no external physical damage was observed to the microorganisms, and they proposed that *C. muelleri* cells were damaged internally.

Wilkinson et al. (2016) investigated the effects of small-scale turbulence on physiological responses and growth rate of *Microcystis aeruginosa* in an isotropic turbulent flow generated by two submersible speakers each behind a fixed mesh grid. Using planar-PIV they measured the spatio-temporally resolved velocity field in four different planes and quantified the turbulent characteristics. They concluded that the growth rate of *M. aeruginosa* was marginally affected by turbulence when exposed to similar conditions to those of Lake Minnetonka, where their culture was isolated.
Xiao et al. (2016) studied the effects of small scale turbulence generated in a stirring apparatus on the physiological and morphological responses of the cyanobacteria *Microcystis flos-aquae* and *Anabaena flos-aquae*. They carried out their experiments in still water and turbulent flows with an intensity range varied from $10^{-3}$ to about $10^{-1}$ m$^2$s$^{-3}$ to cover the range of the turbulence intensities experienced by cyanobacteria in their natural habitat. They cited the book “The Ecology of Phytoplankton” by Reynolds (2006) as the reference for the range of dissipation rate experienced in nature by cyanobacteria. However, in accordance with the reference (Reynolds, 2006) and other references (listed in table 2.1) no intensity higher than about $10^{-4}$ m$^2$s$^{-3}$ was reported in the natural habitat of cyanobacteria.

Using ANSYS software they characterised the flow and computed the turbulence parameters. They observed a generally decreasing trend in the length of *A. flos-aquae* when the turbulence dissipation rate increased. However, for each range of initial filament length the trend was different. For example, they reported that the length of short filaments increased, whereas that of long filaments decreased. They propose that the decrease in filament length was associated with mechanical damage. However, the literature they cited regarding this matter was about the effect of turbulence on dinoflagellates (not cyanobacteria). Dinoflagellates are considered to be the most sensitive of all phytoplankton groups to turbulence (Thomas and Gibson, 1990).

As mentioned in section 2.3.1, one of defence mechanisms of cyanobacteria is to produce a mucilaginous sheath that may prevent physical damage to the organisms. This is a well-known phenomenon for microbiologists and has been widely investigated by them. However, to our knowledge none of the studies that have investigated the effects of turbulence carried out by civil and environmental engineers, oceanographers and marine scientists have taken the existence of this protective envelope into consideration when reporting mechanical damage.
2.5.1 The issues associated with previous investigations of mechanical damage to microorganisms

Based on the reviewed studies that were mentioned and more that were not mentioned here (cited by Hondzo and Lyn 1999; Mitsuhashi et al. 1995; Moisander et al. 2002; Thomas and Gibson 1990; Warnaars and Hondzo 2006), small-scale turbulence can affect the growth rate of microorganisms. Moreover, it is largely concluded that excessive levels of turbulence inhibit the growth of waterborne microorganisms. Despite the existence of only anecdotal evidence and sometimes opposite trends, many hold the view that the possible mechanism of the inhibited growth is the occurrence of mechanical damage to the microorganisms. There are a few points that can justify the existence of the sparse evidence of mechanical damage:

- The sensitivity to turbulence and tolerance of strain rate and the resulting forces are strongly genera and species related (Moisander et al., 2002). Some microorganisms can be damaged by the strain rates in flows (Hondzo and Lyn, 1999), whereas some do not ‘feel’ the turbulence (Thomas et al., 1984a,b,c, 1986). Therefore, no general conclusion can be drawn for all microorganisms.

- Initial health conditions of the microorganisms before the experiments can play a profound role in the results (Moisander et al., 2002). For instance, a developed protective mucilaginous sheath that has not been taken into consideration in previous works may also protect the sheath-producing microorganisms from any potential damage caused by fluid flows.

- Previous studies relied on surrogate estimates of mean quantities of turbulent flows such as dissipation rate and the resulting strain rate. Although considering such mean estimates is crucial, they do not account for the spatio-temporally resolved intermittent structure of small-scale turbulence. Figure
Figure 2.4: An example of a strain rate time-series and its sudden and violent strain rate events measured in a turbulent flow by the Fluid Mechanics Group at the University of Melbourne featuring where the instantaneous rate of strain ($\dot{\gamma}$) is divided by its mean value ($\bar{\gamma}$).

2.4 shows a typical strain rate time-series and exemplifies its sudden and violent strain rate events measured in a turbulent flow by the Fluid Mechanics Group at the University of Melbourne wind tunnel. Therefore, any potential mechanical damage, if it occurs, may be associated with sudden and violent events that happened at different physical points.

- Some of the facilities such as Taylor-Couette can lead to obtaining biased results in regards with making a decision whether the damage to cultures was due to the strain rate of the flow or by striking the microorganisms to the wall of the device or collision of the cultures (Warnaars and Hondzo, 2006). In addition, although some researchers generated laminar flows in this facility, due to the relatively large size of the agglomeration of the cultures (compared
to the gap between the cylinders), the flow was not perfectly the same as the flow with the absence of the cultures. Thus there is still a possibility of the collision of the cultures to the solid surface of the device. Figure 2.5 exemplifies this situation by comparing the sizes of the agglomerates (a few centimetres) compared to the 1.41 cm gap size (not shown). In addition, laminar flows in Taylor-Couette facilities lack a spatially uniform strain rate.

- The conclusion regarding the mechanical damage to the filaments was based on consideration of the morphology or viability of the sub-samples from high throughput microorganisms before and after experiencing long-term turbulence. No direct observation has been carried out to monitor the single cells/filaments of the microorganisms to observe and judge the occurrence of mechanical damage.

### 2.5.2 Present strategy to investigate the potential mechanical damage to cyanobacteria

Micron-sized phytoplankton are usually much smaller than the Kolmogorov length scale (Kolmogorov, 1941) that represents the smallest size of eddies in a turbulent flow. The Kolmogorov length scale is generally agreed to be approximately a
few millimetres for natural environments (Sullivan et al., 2003; Warnaars and Hondzo, 2006; Whitton and Potts, 2000). Therefore microorganisms are exposed to a laminar and spatially uniform straining flow similar to the pure straining flow generated in a Taylor’s four-roll mill (Whitton and Potts, 2000), thereby enabling the researchers to use laminar flow to investigate the effect of strain rate on waterborne microorganisms (Moisander et al., 2002; Warnaars and Hondzo, 2006; Whitton and Potts, 2000).

In this study, in order to observe the effect of the strain rate of the flow on *Anabaena circinalis* filaments, we used laminar flows with spatio-temporally uniform strain rates, thereby avoiding the non-uniformity of the strain rate in turbulence. To establish a spatio-temporally strain rate flow we use a cross-slot type microfluidic device equipped with an advanced real-time control system to immobilise micron-sized target objects in the flow. This enables us to monitor single filaments of *Anabaena circinalis* under a uniform strain rate in stagnation point flows in real-time. Hereafter we review the literature related to this method.

### 2.6 Stagnation point flow

There exist different techniques to enable one to produce extensional flows. These include changing the cross-sectional area of the flow using contraction or expansion (Boger, 1987), thinning fluid filaments using jetting or dripping (Yarin, 1993), and bifurcating the flow (Taylor, 1934) using two opposed laminar streams (stagnation point flow). Amongst the techniques mentioned to produce extensional flows, generating the stagnation point flow has the advantage of the existence of the spatially uniform strain rate. Moreover, the stagnation point enables one to study the deformation of an object at a certain point (i.e. stagnation point). Here we explain two facilities that can generate such flows.
2.6.1 Four-roll mill

Generating a stagnation point flow started with the invention of the so-called four-roll mill by G. I. Taylor in 1934. The apparatus comprises four symmetrically positioned rollers that are rotated in a coordinated manner in a container. A four-roll mill is capable of providing a broad class of two-dimensional, homogeneous flows with linear velocity components:

\[
\begin{align*}
  u &= \gamma x + \omega y, \\
  v &= -\omega x - \gamma y,
\end{align*}
\]

(2.3)

where \( \gamma \) and \( \omega \) are the constants that are the functions of angular speed and direction of the rotation of the rollers. For a particular direction of the rotations of the rollers (schematically shown in figure 2.6), a pure straining stagnation point flow can be produced in a four-roll mill. In this case \( \omega \) is zero and the velocity equations 2.3 are simplified to:

\[
\begin{align*}
  u &= \gamma x, \\
  v &= -\gamma y,
\end{align*}
\]

(2.4)

where \( \gamma \) is the flow strain rate.

A large number of researchers have measured and characterised the flow in four-roll mills (Andreotti et al. 2001; Higdon 1993; Lagnado et al. 1985; Lagnado and Leal...
Lagnado et al. (1984) and have investigated the dynamics of single droplets in the stagnation point flow generated in four-roll mills (Bentley and Leal, 1986; Innings et al., 2005; Milliken and Leal, 1991; Ramaswamy and Leal, 1999). Lagnado and Leal (1990) used fluid visualisation to study the motion of a mixture of 90% glycerol in water. They studied the effects of Reynolds number on the stability of the flow in a four-roll mill. They concluded that as the Reynolds number increases, a significant three-dimensional secondary flow arises near the centre of the top and bottom walls of the container. The flow in the middle sections of the four-roll mill may remain two-dimensional by increasing the Reynolds number. However, at high Reynolds numbers the flow becomes completely unstable inside the container.

Andreotti et al. (2001) investigated the interaction between the strain rate in a four-roll mill. They showed that the pure straining flow becomes intrinsically unstable through a supercritical bifurcation to form an array of counter-rotating vortices aligned in the stretching direction.

They also reported that the shape of the streamlines (when the flow is laminar) does not change much as the angular speed of the rollers increases. It was found that the strain rate varies proportional to the rollers frequency $f_{4rm} = 2\pi\Omega$, where $\Omega$ is the angular speed of the rollers. In particular, the velocity gradients (strain rate) in the central region scale as $f_{4rm}$:

$$\frac{du}{dx} = -\frac{dv}{dy} = \gamma = \kappa f_{4rm}. \quad (2.5)$$

They reported that the proportional coefficients were $\kappa = 1.4$ for a mixture of 10% glycerol in water and $\kappa = 2.7$ for the 99% Glycerol. These values were approximately a quarter of that ($\kappa = 5.1$) found by Taylor (1934). However, there is no report of coefficients of proportionality for water in previous work.
Experiments in water are crucial in our case since the algae would not survive in a medium other than water. Furthermore, experiments with water are more susceptible to instability due to its reduced viscosity compared to other fluids, such as glycerol (to be explained in appendix A).

Due to the existence of the moving elements in a four-roll mill, manufacturing a micron-sized four-roll mill is most challenging. Also, producing a steady hyperbolic flow at high rotational speed and high strain rates is limited. This restriction stems from the growing Dean vortices from the top and bottom of a four-roll mill which make the extensional flow unstable (Andreotti et al., 2001; Dean, 1928). Moreover, due to the low critical Reynolds number in a four-roll mill, it is necessary to use highly viscous fluid such as glycerol (Andreotti et al., 2001; Lagnado and Leal, 1990). Another issue caused by the low critical Reynolds number in a four-roll mill is the limitation in increasing the flow strain rate, and the maximum strain rate that can be reached in a four-roll mill was reported to be about 7 s\(^{-1}\) (Andreotti et al. 2001 and appendix A). These facts limit studying the dynamics of micron-sized objects such as waterborne microorganisms in this type of flow.

In appendix A we report a stand-alone experiment in a miniature four-roll mill, which a parametric study and the possibility of straining a micron-sized object in water flows in this device were studied. However, due to the issues mentioned in the preceding paragraph, we eventually used another device (a cross-slot type microfluidic device) to generate the straining stagnation point flow.

### 2.6.2 Cross-slot type microfluidic device

A cross-slot type microfluidic device, or simply a cross-slot microchannel, consists of two micron-sized inlet channels with two converging streams and two outlet channels with two diverging streams. Both outlet channels (extensional axis) set out perpendicularly to the inlet channels (compressional axis) in a horizontal plane (schematically shown in figure 2.7). In the intersection of the cross-junction, where
the two laminar flows meet each other, a two-dimensional straining flow with a zero-velocity (stagnation) point is generated. The flow generated in the intersection of the cross-slot is similar to that of the Taylor’s four-roll mill with hyperbolic streamlines and the velocity field of equation 2.4 \( (u = \gamma x \text{ and } v = -\gamma y) \), where \( \gamma \) is the strain rate of the flow.

![Diagram](image)

**Figure 2.7:** Three-dimensional schematic of the cross-slot channel. The dimensions represent the dimensions of the channel to be used in this work.

These microfluidic devices can be used as extensional rheometers (Haward and McKinley, 2012; Haward et al., 2011; Sharma et al., 2015). The instability of the flows of non-Newtonian fluids at high flow rates limits applying very high strain rates in this type of rheometer. Arratia et al. (2006); Haward and McKinley (2013); Rocha et al. (2009) and Schroeder et al. (2003) investigated the instability of Non-Newtonian flows such as polymer solutions and biofluids. Arratia et al. (2006) studied the instability of extensional flow in a cross-slot microchannel, where they only observed the unstable flow while using a non-Newtonian polymer solution, and a stable hyperbolic flow while using a Newtonian fluid within the range of their Reynolds number. Rocha et al. (2009) numerically studied visco-elastic fluid flows in this device and showed that rounding the corners marginally affected the instability of the flows.
Another application of a cross-slot type microfluidic device is enabling the characterisation and observation of deformation and breakup of a bubble or droplet (Ulloa et al., 2014), confining a micron-size bead (Johnson-Chavarria et al., 2011; Tanyeri and Schroeder, 2013; Tanyeri et al., 2010, 2011a), a motile bacterium (Johnson-Chavarria et al., 2014), stretching a DNA polymer strain (Kantsler and Goldstein, 2012; Li et al., 2015; Tanyeri et al., 2010; Xu and Muller, 2011) and phenotyping of cancerous cells (Gossett et al., 2012; Henry et al., 2013) in stagnation point of the flow.

2.7 Immobilising and stretching single objects with a specific focus on cross-slot microchannels

Over the last three decades, recent advances in microfluidic systems have enabled researchers to trap and immobilise nano- and micron-sized objects such as single micro-particles, cells and macro-DNA molecules using different types of microfluidic traps such as magnetic (Gosse and Croquette, 2002; Lee et al., 2004), optical (Ashkin et al., 1986; Grier, 2003; Yang et al., 2009), acoustic (Bernassau et al. 2014; Hertz 1995; Qiu et al. 2014) and hydrodynamic traps (Tanyeri et al., 2010, 2011a).

Amongst the traps mentioned the hydrodynamic trap solely utilises hydrodynamic forces for confinement of a micron-sized object in a flow, whereas the others are dependent on external forces such as magnetic, acoustic and optical. Therefore, a hydrodynamic trap is the best option to conduct biological experiments when similarity of the flow to their natural habitat is required. Hydrodynamic traps are largely divided into two categories, contact-based techniques (Di Carlo et al., 2006; Tan and Takeuchi, 2007) and noncontact-based techniques. The former methods are based on confinement of an object against physical obstacles, whereas the latter
methods are based on the trapping of an object in a stagnation point flow (similar to the Taylor’s four-roll mill), or micro-vortices and eddies (Lin et al., 2008). As mentioned before, amongst the trapping techniques we chose a noncontact-based hydrodynamic trap where single objects are trapped in the stagnation point flow in a cross-slot microchannel (with a spatially uniform strain rate), as we intend to solely investigate the effect of the strain rate (in a straining flow) on waterborne microorganisms.

As mentioned above, the first hydrodynamic trap was in a four-roll mill, which is still used to trap millimetre-sized objects. In the 1990s, with the emergence of microfluidic devices, cross-slot microchannels were used to trap micron-sized objects such as DNA molecules. However, this was limited to short-term confinement of the target (Perkins et al., 1997) as the stagnation point in this flow is a semi-equilibrium stable point (to be explained in section 5.1.1). Later, relatively longer confinement of single objects was achieved using human-facilitated control systems in cross-slot microchannels (Dylla-Spears et al., 2010; Schroeder et al., 2003, 2004; Xu and Muller, 2011). For instance, Schroeder et al. (2004) studied the hydrodynamic interaction of flexible polymer chains in the planar extensional flow and they found that conformation-dependent resistivity of the chains can be used to predict the polymer behaviour in the coarse-grained polymer models. Ulloa et al. (2014) measured the velocity field in the cross-junction of a microchannel at a single low flow rate and strain rate, and they studied the deformation of a drop in the stagnation point of the flow.

Over the last several years, using an image-based active control system, long-term trapping of a single micron-sized object became possible. Schroeder and coworkers (Johnson-Chavarria et al., 2011, 2014; Latinwo et al., 2014; Li et al., 2015; Shenoy et al., 2015; Tanyeri and Schroeder, 2013; Tanyeri et al., 2010, 2011a) developed an image-based micro-hydrodynamic trap in the straining flow of a cross-slot microchannel for high-resolution confinement of single micron-sized objects. To tackle the Brownian motion and to achieve long-term confinement of micron-sized
beads, Tanyeri et al. (2010) implemented an on-chip membrane valve (filled with a pressurised gas) on one of the outlets of the cross-junction of the microfluidic device to control the position of the stagnation point (to be explained in section 5.1). Johnson-Chavarria et al. (2011) demonstrated the fabrication of the double-layer microfluidic device used in this type of microfluidic trap and visualised how to operate the trap. Tanyeri et al. (2011a) comprehensively presented the design and implementation of the hydrodynamic trap and characterised the effect of various parameters used in fabrication of the microfluidic device and the dimensions of the device on the time response of the membrane valve. Tanyeri et al. (2011b) used the same on-chip membrane valve and the control system to produce a fluid flow in a microfluidic device similar to the current in a Wheatstone bridge circuit. Kantsler and Goldstein (2012) used the same trapping system, however, instead of the on-chip membrane valve, they controlled the position of the stagnation point by directly controlling the pressure differences of one of the outlets. They studied the dynamics of single actin filaments in the straining flow at strain rates between 0.1-1 s$^{-1}$, which were enough to stretch the filaments. Tanyeri and Schroeder (2013) added an extra on-chip membrane valve on one of the inlets, in order to manipulate the stagnation point position in two dimensions. The new on-chip valve does not improve the capability of the trap as the stagnation point is an equilibrium stable point along the compressional axis of the straining flow. Johnson-Chavarria et al. (2014) used an adaptive control algorithm to achieve longer trapping and they confined an Escherichia coli cell, to investigate the effect of cell growth environment by observation of the cell growth dynamics, intracellular diffusion proteins and gene expression. Latinwo et al. (2014) and Li et al. (2015) used the same type of microfluidic trap to study non-equilibrium thermodynamics of a polymer solution and stretching circular polymers in the straining flow. Shenoy et al. (2015) characterised the performance of the control system of the trap by using combinations of proportion-integral-derivative controllers to improve the stiffness of the trap. Shenoy et al. (2016) used the same basic concepts of the trapping, in a microfluidic device with more inlets and outlets to produce a double-stagnation point flow.
to simultaneously trap multiplexed particle manipulation (called a Stokes trap). Alicia et al. (2016) modified an image-based micro-hydrodynamic trap to dynamically establish concentration gradients without increasing the shear rate.

In all of the studies mentioned the experiments were conducted at low strain rates (up to 1 s\(^{-1}\)), as the aim of their experiments were immobilising a target, such as fluorescent beads, single cells or stretching soft and very flexible DNA molecules.

Recently, cell phenotyping based on cell deformability using the cross slot microchannels has become popular (Cha et al., 2012; Gossett et al., 2012; Henon et al., 2014; Henry et al., 2013). Gossett et al. (2012) and Henry et al. (2013) applied extremely high strain rates (about 10\(^5\) s\(^{-1}\)) for a high throughput of 2000 cell/s. They used an inertial focusing method to focus the cell on the extensional axis. Using a high speed camera, they observed and characterised the cell deformability at the cross-slot junction. The residence time of the sample was O(10\(^{-5}\) s) and this method is not suitable for exposing the objects to a straining flow for a long time. By modifying the optics and manufacturing a glass cross-slot microchannel, De Loubens et al. (2015) managed to three-dimensionally image the cross slot junction from two sides and calculate the Poisson ratio of human serum albumin capsules.

### 2.8 Fluid mechanics measurement in cross-slot microchannels

Regarding the measurement at the cross-slot junction, some researchers obtained the velocity field by measuring the velocity of a few tracked particles and substituting their velocity in the velocity equations (equation 2.4) of the pure straining flow (Tanyeri et al., 2010). Others conducted micro-PIV measurements to measure the more accurate velocity field and the strain rate (Alicia et al., 2016; Pathak
and Hudson, 2006) and some numerically simulated the flow in the cross junction using commercial software (Dylla-Spears et al., 2010).

Using a numerical simulation in COMSOL, Dylla-Spears et al. (2010) showed that the strain rate changes less than 5% within a radius of $w/16$ in the vicinity of the stagnation point, where $w$ is the width of the channel. Pathak and Hudson (2006) conducted micro-PIV to measure the velocity field of the flow of a non-Newtonian fluid and reported the strain rate changes less than 5% within a radius of $w/4$.

In this study, prior to exposing the cyanobacteria to the straining flow, we systematically measured the velocity fields and computed the strain rates at different flow rates using micro-PIV and fully characterised the flow to identify the uniform strain rate region. Moreover, we later measured the velocity field around the filaments of cyanobacteria while they were trapped in the stagnation point. This enabled us to calculate the stretching and compressing forces on the filament. It also opens new vistas of characterising mechanical properties of micron-sized objects. No such measurements were carried out when a micron-sized object was confined at the stagnation point and all previous studies were based on the measurement of the ideal flow with the absence of the trapped object.

### 2.9 Micron-resolution Particle Image Velocimetry (micro-PIV): a brief review

Particle Image Velocimetry (PIV) is a measurement technique in fluid mechanics in which velocity fields are instantaneously measured by measuring the displacements of numerous fine particles in a flow. The particles are assumed to have the same density as the flow, and to be sufficiently small to follow the flow with no influence on it. The flow and the seeding particles are commonly illuminated using a pulsed laser light sheet.
In PIV, two successive images of the flow seeded with tracing particles are recorded with a specified time interval ($\Delta t$) and the images are then spatially subdivided into different regions known as interrogation windows. By cross-correlating the interrogation windows the local displacement ($\Delta x$) corresponding to each interrogation window is determined and the velocity is computed by $u = \frac{\Delta x}{\Delta t}$. The basic principles and comprehensive literature review of PIV technique were presented by Adrian (1991); Adrian and Westerweel (2011); Raffel et al. (2013); Wereley and Meinhart (2010); Westerweel et al. (2013).

Micron-resolution Particle Image Velocimetry (also known as micro-PIV or µ-PIV) is a fundamentally similar measurement technique to PIV used to measure the velocity in microfluidic devices, with the resolution ranging from 0.1 to 100 microns. However, due to optical and mechanical limitations in microfluidics, different seeding particles, illumination, processing and pre- and post-processing are required. Micro-PIV was first introduced by Santiago et al. (1998) who conducted a micro-PIV measurement in a microchannel with the resolution of 10 $\mu$m, however, traces of fluorescent microscopy can be found in previous works by Brody et al. (1996); Taylor and Yeung (1993) in their particle-streak velocimetry experiments. Following the first micro-PIV article, Meinhart et al. (1999) measured the velocity profile of a laminar flow in a rectangular microchannel with the resolution of one micron. Later, the same group presented a theoretical expression for the micro-PIV depth of measurement and the effect of the seeding density on the signal-to-noise ratio (Meinhart et al., 2000a). They also introduced time-averaging techniques in micro-PIV recordings to increase the spatial resolution, where low image density micro-PIV images are cross-correlated (Meinhart et al., 2000b). Three techniques “Average Velocity Method”, “Average Image Method” and “Average Correlation Method” were introduced. Apart from the time-averaging techniques, more advanced processing techniques such as Central Difference Interrogation (CDI) and window deformation methods were later presented by Wereley et al. (2002) in order to reduce both PIV and micro-PIV bias errors. They implemented the methods
in the micro-PIV measurements in the straining flow in a four-roll mill and the flow in a micronozzle. Gui et al. (2002) studied the effect of the digital filtering on background noise micro-PIV recordings and found that a combination of a $3 \times 3$-pixel smooth filter and an unsharp mask enhances the accuracy of the results by reducing the signal-to-noise ratio. Bourdon et al. (2006) modified the theoretical expression for depth of correlation (previously stated by Olsen and Adrian 2000) for high numerical aperture oil immersion objective lenses.

The articles mentioned above used the original micro-PIV technique known as classical micro-PIV (Wereley and Meinhart, 2010). Today, recent technological advances in imaging equipment and light sources enable the investigators to conduct more complex measurements. As three-dimensional micro-PIV is not the main focus of this work, we direct the reader to the articles that used different micro-PIV techniques to measure the three-dimensional velocity field, such as scanning multiple two dimensional planes (Pommer, 2007), digital holography (Yang and Chuang, 2005), confocal fluorescent microscopy (Mogi and Sugii, 2016; Park et al., 2004) and three-dimensional measurements of velocity field by a single camera using a defocused technique (Cierpka et al., 2010).

### 2.10 Summary and research questions

By reviewing incidents caused by harmful cyanobacteria throughout recent history, we have highlighted the importance of conducting a cyanobacteria-related investigation. The sparse evidence of the effects of small-scale turbulence on microorganisms and the additional anecdotal evidence of mechanical damage to cyanobacteria motivated us to carry out systematic research to observe and quantify the potential damage to one of the species (Anabaena circinalis) of these deleterious microorganisms.
In order to avoid the limitations of previous studies, such as unsteadiness of the turbulent flows, non-uniform strain rates of the flows and no direct observation of the filaments, we chose an entirely different approach. In our approach instead of exposing a large number of filaments to a non-uniform strain rate flow, we expose each filament of *A. circinalis* to a uniform strain rate flow in a cross-slot type microfluidic device. Using an advanced image-based active control technique (previously developed by other researchers) enables us to directly monitor the filaments under strain rate. Moreover, by modifying this technique we propose and conduct a novel experiment to simultaneously measure the velocity field using micro-PIV, when the *A. circinalis* filaments are trapped.

In summary, from a microbiological point of view we intend to observe potential mechanical damage to *A. circinalis* filaments that may be caused by the strain rate of the flow. From a fluid mechanics point of view we intend to improve the applicability and capability of single object trapping in the straining flow by integrating this technique with simultaneous micro-PIV measurement.
Chapter 3

Fabrication of microfluidic devices

To manufacture cross-slot type microchannels with on-chip membrane valves, double-layer microchannels were fabricated using soft-lithography. All of the fabrication steps described in this chapter were performed by the author at the Melbourne Centre of Nanofabrication (MCN) at the Victorian Node of the Australian National Fabrication Facility (ANFF).

3.1 Basics of soft-lithography

Soft-lithography is the most common technique of microfluidic device fabrication. It is a method of casting and fabricating elastomeric materials such as polydimethylsiloxane (PDMS) on a master mould in micro- or nano-metre scales with high fidelity (order of 10 nm). The PDMS used in soft-lithography consists of two separate components, a base and a curing agent (cross-linker), mixed together prior to the casting process. Different ratios of the base and cross-linker give different final PDMS physical properties, particularly stiffness (Duffy et al., 1998; McDonald and Whitesides, 2002).
Prior to soft-lithography, a master mould is fabricated consisting of the designed imprinted features (positive relief) of the microchannels on a substrate. Master moulds are usually fabricated using contact photo-lithography with a photoresist on a silicon substrate (wafer). In order to fabricate a master mould, the substrate and the negative photoresist material, are spin-coated to reach the required thickness. Afterwards, ultraviolet (UV) light is transmitted through a high resolution transparency photo-lithographic mask. Consequently, the areas of the photo-resist (microchannel features) that were exposed to the UV light are linked to the wafer. The photo-resist usually used in this type of fabrication is SU-8 (an epoxy-based negative photo-resist) (Duffy et al., 1998; McDonald and Whitesides, 2002; McDonald et al., 2000).

3.2 Fabrication of double-layer microfluidic devices

The fabrication protocol of a double-layer microfluidic device (a cross-slot microchannel with an on-chip membrane) valve is detailed. Hereafter the term ‘fluidic’ is used to note the components corresponding to the cross-slot microchannel (e.g. fluidic substrate/wafer, fluidic mask and fluidic PDMS layer), while the term ‘control’ is used to note the components corresponding to the on-chip membrane valve (e.g. control substrate/wafer, control mask and control PDMS layer).

Figure 3.1 shows the flowchart of the process of fabricating a double-layer microfluidic device (excluding maskless photo-lithography) used in this work: fabricating master moulds, casting PDMS on the masters and bonding the PDMS replica to a glass substrate.
(a) Contact-lithography: UV light is emitted through the negative photo-lithographic masks (section 3.2.2.4) to the spin-coated (section 3.2.2.2) and soft-baked (section 3.2.2.3) SU-8 photoresists.

(b) Wafers are post-baked to cure the exposed SU-8 (section 3.2.2.5) and then developed using SU-8 developer (section 3.2.2.6) to finalise the fabrication of the master moulds. The moulds are then silanized to be prepared for PDMS casting (section 3.2.3.1).

(c) PDMS casting: Mixtures of PDMS base and its curing agent (1:15 for the fluidic layer and 1:5 for the control layer) are degassed (section 3.2.3.2) and poured on the moulds (section 3.2.3.2). The fluidic master mould is spin-coated to reach a PDMS layer with a 110 µm thickness, while a thick layer of PDMS mixture is poured over the control mould without spin-coating. Afterwards, both of them are soft-baked (section 3.2.3.3).
(d) The soft-baked PDMS replica is peeled off the control mould and hole punched (section 3.2.3.5). At this stage fluidic layer is remain unchanged.

(e) The PDMS replica that was peeled off the control layer is aligned on the fluidic layer (section 3.2.3.5) to be post-baked (section 3.2.3.6).

(f) The post-baked PDMS slab including the fluidic layer cured to the control layer are peeled off the fluidic mould and the fluidic layer ports are hole punched (section 3.2.3.6).

(g) Plasma-cleaning: The PDMS replica is plasma-cleaned and irreversibly bonded to a glass substrate to seal the microfluidic device. Finally, the microfluidic device is baked overnight to finalise the bonding (section 3.2.3.7).

Figure 3.1: The flowchart of the process of fabricating a double-layer microfluidic device (excluding maskless lithography): fabricating masters (section 3.2.2), casting PDMS on the masters and bonding the PDMS replica to substrates (section 3.2.3).
3.2.1 Fabricating photo-lithographic mask using maskless lithography

In this subsection fabrication of negative photo-lithographic masks is described.

3.2.1.1 Designing and drawing microchannels

The three different cross-slot microchannel designs shown in figure 3.2 were two-dimensionally drawn using a CAD program (Layout Editor) and later fabricated. The black features indicate the fluidic channels and the red features indicate control channels. The channels in figures 3.2a and 3.2b were designed and used in previous studies by Tanyeri et al. (2010, 2011a) and Johnson-Chavarria et al. (2011, 2014) with four and two inlets, respectively. The channel in figure 3.2c, designed in the present work, has only one inlet. The advantage of the first design (figure 3.2a) is the position of the sample injection/focusing port, which helps samples to be focused on the centre line of the cross-junction, thereby allowing for easier trapping at the stagnation point. However, the greater the number of inlets, the greater the likelihood of a defect in the access ports. Moreover, the possibility of leakage and formation of bubbles in the access ports is higher in multiple inlet channels compared to the present design (figure 3.2c).
Chapter 3. Fabrication of microfluidic devices

(a) Four inlets, designed and used by Tanyeri et al. (2010, 2011a) and Johnson-Chavarria et al. (2011).

(b) Two inlets, designed and used by Johnson-Chavarria et al. (2014).

(c) One inlet (new design in the present work).

Figure 3.2: Three different designs of cross-slot microchannels with different number of inlets that are fabricated in this work (black: fluidic channels, red: control channels).
3.2.1.2 Printing microchannel features on negative photo-lithographic masks

The first step in fabricating a microfluidic device is to create a negative photo-lithographic mask with transparent microchannel patterns. In this work, using the maskless lithography technique, two different photo-lithographic masks were manufactured in order to fabricate fluidic and control moulds.

As many channels as possible were fitted into a four inch diameter circle (figure 3.3), as later these channels were fabricated on four inch diameter silicon wafers. A centimetre gap was allocated between the features and the circle edge to guarantee the production of a uniform thickness on top of the features by spin-coating (described in section 3.2.2.2).

The channel drawings were converted to transparency and printed on a five inch square chrome photo-mask using a high resolution (4.88 nm) and accuracy ($\pm$200 nm) mask writer (XF 100 Xpress, Intelligent Micro Patterning). The channel features were transparent and the other areas of the mask were covered with a chrome-metal absorbing film (figure 3.4).
3.2.2 Fabricating master moulds using contact-lithography

In this section the fabrication of a positive relief SU-8 structure on silicon substrates using contact-lithography in a class 100 clean room is explained.

The following conditions were applied to obtain 40 µm and 130 µm thickness for fluidic and control moulds, respectively. These thicknesses define the height of the channels.

3.2.2.1 Preparing silicon substrates

Two factory-cleaned, four inch diameter silicon wafers (one for the fluidic and the other for the control master moulds) were used to receive SU-8 photo-resist. Where wafers are not factory-cleaned, acetone and isopropyl alcohol (IPA) can be used to clean the wafer and then it can be dried by pressurised gas and placed on a clean hot-plate for one minute to remove any moisture (Johnson-Chavarria et al., 2011), however, acetone and IPA may stain the wafers.
3.2.2.2 Pouring, spin-coating the SU-8 on the silicon wafers

To produce the desired thickness of SU-8 layers on the wafers, each wafer was placed into a spin-coater and aligned using a Laurell wafer alignment tool. Subsequently, a puddle of SU-8 3050 and SU-8 100 (MicroChem) were poured and spin-coated on the silicon wafers at the conditions listed in table 3.1.

| Photo-resist Viscosity Speed Duration Thickness |
|-----------------|------------|-----------|-------|
| Fluidic wafer   | SU-8 3050  | 12250     | 4000  | 30    | 40     |
| Control wafer   | SU-8 100   | 51500     | 1750  | 30    | 130    |

To reduce wasting expensive photo-resist substance during spin-coating, only half of the area of the wafers was covered with the SU-8. To avoid bubble formation during the pouring process, the SU-8 bottle was held as close as possible to the wafer.

3.2.2.3 Soft-baking of SU-8 on the silicon wafers

To remove the solvent from, and densify, the SU-8, soft-baking was applied. It is usually conducted as two steps, first at 65°C and then at 95°C, on clean contact hotplates. The duration of each step depends on the thickness of the SU-8 layer. The conditions used in this work are listed in table 3.2.

<table>
<thead>
<tr>
<th>Photo-resist</th>
<th>Step one @ 65°C</th>
<th>Step two @ 95°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluidic wafer</td>
<td>SU-8 3050</td>
<td>3</td>
</tr>
<tr>
<td>Control wafer</td>
<td>SU-8 100</td>
<td>20</td>
</tr>
</tbody>
</table>
3.2.2.4 Exposing UV light source through the photo-mask

To cure and link the SU-8 to silicon, UV light was emitted through the transparent features of the masks to those areas which later shape the microchannel structures. Wafers with the corresponding masks on top, with no interface and gap in between, were placed in a UV flood light source (ABM, resolution = 5 \mu m) and were exposed to UV light with the intensity of 150 mJ/cm\(^2\) at 365 nm for the fluidic and 400 mJ/cm\(^2\) for the control wafers, respectively (schematically shown in figure 3.1a).

3.2.2.5 Post-exposure baking the SU-8 moulds

To complete polymerisation, both wafers were post-baked on the clean hotplates in two steps and the conditions are described in table 3.3. Post-baking was followed by one minute cooling at the room temperature.

<table>
<thead>
<tr>
<th>Photo-resist</th>
<th>Step one @ 65°C (Min)</th>
<th>Step two @ 95°C (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluidic wafer</td>
<td>SU-8 3050</td>
<td>1</td>
</tr>
<tr>
<td>Control wafer</td>
<td>SU-8 100</td>
<td>2</td>
</tr>
</tbody>
</table>

3.2.2.6 Developing the SU-8

To remove the un-exposed, un-cured and unwanted SU-8, the wafers were soaked in SU-8 developer (Propylene glycol methyl ether acetate (PGMEA), MicroChem) until the features were revealed. The wafers were then rinsed with acetone and DI water until all the unwanted liquids were washed off the moulds (schematically shown in figure 3.1b). This step was followed by drying with pressurised nitrogen.
3.2.2.7 Measuring fabricated structures

To ensure the desired height of the channels were reached, the height of the imprinted structures on the silicon substrates (the positive relief in figure 3.5) were measured under a one nano-meter resolution profilometer (Ambios XP200 Profiler). The heights of positive relief structures of fluidic and control substrates were 40 µm and 130 µm, as predicted.

![Figure 3.5: Left: Si/SU-8 master moulds for (Right) fluidic channel (left) control channel.](image)

3.2.3 PDMS casting

In this section PDMS casting against the master moulds (fabricated with the protocol in section 3.2.2) is explained.

3.2.3.1 Silanizing the master moulds

To prepare the surface of the wafers for PDMS casting, silanization was applied. Silanization is the process during which silicon becomes hydrophobic through self-assembled monolayers by covering the surface of silicon with approximately a few
hundred nanometer thickness of silanizer (Ting et al., 2011). This step assists in stripping the PDMS from silicon, as silicon is a naturally hydrophilic polymer.

In this experiment, for silanization, the wafers were transferred into a desiccator along with a pipette head funnel containing 20 µL of silanizer (trichlorosilane). Then they were placed under a vacuum for 30 minutes, and then left in the desiccator for 24 hours. A 24 hour silanization enables the mould to be used four or five times, then re-silanization is required. Due to the durability of the silicon wafers, they can be used indefinitely and do not fail, unless broken (McDonald et al., 2000).

### 3.2.3.2 Mixing and degassing the base and the cross-linker

To cast and solidify the naturally liquid PDMS that comes in two parts (base and cross-linker), both components are mixed and then baked. Different ratios of the base to cross-linker lead to different stiffnesses of the PDMS slab (Wang et al., 2014). The lower the ratio of base to cross-linker used, the stiffer the PDMS obtained.

In accordance with Tanyeri et al. (2011a) and Johnson-Chavarria et al. (2011), in this work PDMS (Sylgard 184 silicone kit, Dow Corning) and curing agent were mixed using the conditions described in table 3.4.

<table>
<thead>
<tr>
<th>Table 3.4: SU-8 spin-coating parameters.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base (gr)</td>
</tr>
<tr>
<td>Fluidic layer</td>
</tr>
<tr>
<td>Control layer</td>
</tr>
</tbody>
</table>

Both mixtures were placed in a degassing desiccator for 30 minutes to ensure all the bubbles were removed from the liquid PDMS mixtures.
3.2.3.3 Spin-coating and soft-baking of the mixtures

For the spin-coating and soft-baking of the mixtures, the parameters used by Tanyeri et al. (2011a) and Johnson-Chavarria et al. (2011) were implemented. To make a 66 µm thick membrane (the thickness of the fluidic layer over the SU-8 structures), the fluidic wafer was spin-coated at 750 RPM for 30 sec. Afterwards, to soft-bake the wafers, both wafers (schematically shown in figure 3.1c) were transferred into a convection oven at 65°C for 30 minutes.

3.2.3.4 Measuring the thickness of the membrane valve

To measure the thickness of the PDMS layer under the profilometer, a small part of the PDMS was cut (using a blade) and peeled off the fluidic wafer. This thickness was subtracted from the height of the SU-8 structures (previously measured in section 3.2.2.7) to calculate the thickness of the membrane. The measured thickness was 70 µm.

3.2.3.5 Hole punching the control layer and aligning on the fluidic-layer

After the partial baking, the PDMS replica on the control layer was peeled off slowly from the wafer. The access ports were punched out using a 26 gauge blunt syringe needle (Zephyrotronics), or a 0.75 unicore manual puncher (schematically shown in figure 3.1d). Afterwards, the control PDMS slab was aligned over the fluidic layer under a stereo-microscope (schematically shown in figure 3.1e). Scotch tape was used to remove the statics before aligning the layers. Although statics do not directly affect the channels, removing them results in a cleaner control layer.
3.2.3.6 Post-baking and hole punching the fluidic layer

To fully cure the PDMS layers together, the fluidic wafer with the fluidic and control PDMS slabs on top were placed into the convection oven for 24 hours. Then the cured PDMS slabs were peeled off the wafers and each individual double layer-channel was separated using a razor blade. Afterwards, all the access ports of the fluidic layer were hole punched with the same technique as the control layer (schematically shown in figure 3.1f).

3.2.3.7 Oxygen plasma-cleaning and bonding PDMS slab to glass slides

To form an enclosed microchannel and irreversibly bond the PDMS replica to a piece of glass, each microchannel and a glass slide (70 × 26 × 1 mm, Waldemar Knittel) were placed in a plasma cleaner (Harrick Plasma Cleaner, PDC-002) for two minutes under a vacuum (300 mTorr). Next, the oxidised PDMS surface and the glass slide were brought immediately together and gently pressed by hand. Finally, all the channels (schematically shown in figure 3.1g) were baked for 24 hours to stabilise and finalise the bonding. Figure 3.6 shows a fabricated microchannel.
Figure 3.6: Photograph of a fabricated microfluidic device. The fluidic channel is filled with blue ink, while the control channel is filled with dark red ink.
3.2.4 Connecting the microfluidic device to the outside world

In order to drive the fluid into the microchannel, gas-tight glass syringes (1000 µL and 250 µL, Hamilton) and a programmable syringe pump (Harvard PHD ULTRA) were used. The configuration of the syringes differs for each experiment and is described in the corresponding chapters.

Luer lock adaptors and 24 gauge bent metal tubing were used to connect the perfluoroalkoxy (PFA) tubes to the syringes and access ports of the microfluidic device via PFA tubes (0.020 inch inner diameter (ID), 1/16 inch outer diameter (OD), IDEX), respectively. A micro manual shut-off valve (1/16 inch OD tubing Upchurch) was used to block the injection port when required. The same metal tubing and PFA tubes were used to establish the connection between the microfluidic device and the drain reservoir. To maintain a constant pressure drop the outlet
tubes were of equal length and both were submerged into a 28 mL polypropylene sample container. It should be noted that, for all the experiments, pre-filling the tubes is a key factor to significantly reducing the quantity of bubbles in the flow. Figure 3.7 illustrates a microfluidic device and its connections to the syringes and pump mounted onto a microscope stage.

3.3 Summary

The fabrication protocol of the double layer microchannels using soft lithography has been detailed. Negative photo-lithographic masks were manufactured using maskless lithography. This was followed by the fabrication of master moulds using the contact-lithography technique. The master moulds consist of the SU-8 cured onto silicon substrates. By casting PDMS against the master moulds the microchannel features on the moulds were replicated on the PDMS slabs. Finally, the PDMS replicas were bonded to glass slides to form a sealed microfluidic device using oxygen plasma-cleaning.
Chapter 4

Micro-PIV measurements in cross-slot type microchannel

In this chapter the micro-PIV experiments are described, which are used to measure the velocity field and calculate the strain rate in the cross-slot microchannel. The final objectives of these experiments are to calibrate the strain rate versus flow rate and also to determine spatially uniform strain rate region with velocity profile of \( u = \gamma x \) and \( v = -\gamma y \) in the cross-slot junction. This chapter covers micro-PIV measurements without flow control and alga filaments.

4.1 Basics of micro-PIV

As mentioned in section 2.9, in contrast with macroscopic PIV, micro-PIV has a different configuration for illumination. Due to the small size of the microfluidic devices and the restriction in optical access to the flow, instead of making a laser light sheet the whole flow volume is illuminated. The volume illumination technique results in bright background in the images. The source of this background is the light reflected from the structures of the microfluidic device. To address this issue, wavelength separation is implemented, i.e. illuminating fluorescently dyed
seeding particles by a specific wavelength using a laser or other light sources (e.g. green light). These particles only absorb the specific wavelength of the laser (green light), but they only emit another waveband (e.g. red light). Then by filtering the green light, the camera sensor does not record the reflected lights from the walls nor from the laser. Therefore, it only captures red light from the particles. This technique is known as epifluorescence imaging (Adrian and Westerweel, 2011).

Other difficulties with micro-PIV measurements are the existence of out-of-focus particles caused by volume illumination and the small depth of focus of the microscope objective. Figure 4.1 shows how volume illumination and the small depth of focus of the microscope objective leads to the creation of the out-of-focus particles. The out-of-focus particles considerably reduce the signal-to-noise ratio of the correlation map.

![Figure 4.1: Schematic of volume illumination in micro-PIV.](image)

Using low seeding density and some image processing techniques the out-of-focus particles can be removed from the images (to be described in section 4.7.1 and 4.7.2).
4.2 Imaging setup

A high speed CMOS sensor camera (2000 × 2000 pixels, 12 bits, PCO.dimax HS4), that is able to capture back-to-back images at 2277 fps at full resolution, was coupled with an inverted Nikon microscope side port to record the particle images. The minimum exposure of the camera and the pixel spacing were 1.5 µs and 11 µm, respectively. The default optical interface of the camera was replaced by a C-mount interface to couple the camera with the side port of the microscope through a C-mount 1× relay lens. Due to the large camera sensor (22 × 22 mm²) and using 1× relay lens, vignetting of the images was inevitable. In general, using relay lenses with lower magnifications increases the vignetting and higher magnifications (in this case 2×) can solve the vignetting problem. There exist third party relay lenses (M>1) known as universal DSLR adapters (to be used and explained in section 5.3.1 for the purpose of movie creation). However, due to the aberration that these lenses produce in the images, they are not sufficiently precise or suitable for accurate micro-PIV measurements. However, they are suitable for the purpose of movie creation where no accurate measurements are carried out.

The microscope was equipped with a CFI S Plan Fluor ELWD 20× (NA = 0.45) objective, ideal for fluorescence imaging. In later experiments, it was replaced by a 50× Mitutoyo Plan Apo SLWD infinity corrected (NA = 0.42) objective. To attach the Mitutoyo objective to the Nikon microscope a M26 to M25 adapter was used.

Although using different magnifications leads to different fields of view (FOV) and spatial resolutions (defined by the interrogation window size), we obtained almost the same resolutions. This was due to using the same seeding particles size (1 µm) and applying larger interrogation windows while using higher magnification lens. One can seed the flow with smaller particles to increase the spatial resolution. All the PIV parameters for both cases are listed in table 4.1. However, the values shown and explained in this chapter are from (M = 20×, larger FOV), unless
otherwise stated. In both cases all the final results (velocity field and strain rate) were the same.

Table 4.1: Micro-PIV and experimental parameters

<table>
<thead>
<tr>
<th>Microscope Objective</th>
<th>Nikon</th>
<th>Mitutotyo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Objective magnification</td>
<td>20×</td>
<td>50×</td>
</tr>
<tr>
<td>Numerical aperture</td>
<td>0.45</td>
<td>0.42</td>
</tr>
<tr>
<td>Image size (px)</td>
<td>800 × 800</td>
<td>1360 × 1360</td>
</tr>
<tr>
<td>Optical magnification (µm/px) (section 4.4)</td>
<td>≈ 0.29</td>
<td>≈ 0.55</td>
</tr>
<tr>
<td>Depth of field (µm) (section 4.6)</td>
<td>≈ 2.5</td>
<td>≈ 2.9</td>
</tr>
<tr>
<td>Depth of correlation (µm) (section 4.6)</td>
<td>≈ 7.8</td>
<td>≈ 6.7</td>
</tr>
<tr>
<td>Field of view (µm × µm)</td>
<td>360 × 360</td>
<td>290 × 290</td>
</tr>
<tr>
<td>Number of overlaid images (section 4.7.2)</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>Interrogation window size (px)</td>
<td>32 × 32</td>
<td>64 × 64</td>
</tr>
<tr>
<td>Interrogation windows overlap</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Spatial resolution (µm)</td>
<td>≈ 9</td>
<td></td>
</tr>
<tr>
<td>Particle diameter (µm)</td>
<td>≈ 1</td>
<td></td>
</tr>
<tr>
<td>Seeding</td>
<td>Red fluorescent polystyrene microspheres</td>
<td></td>
</tr>
<tr>
<td>Flow medium</td>
<td>Distilled water</td>
<td></td>
</tr>
</tbody>
</table>

### 4.2.1 Epifluorescence imaging

To illuminate the flow the Epi-fl illuminator of the microscope (excluding the connection port inserted into the microscope body) was removed to provide optical access for laser light. A 532 nm Nd:YAG double-pulsed laser (200 mJ per 5 ns pulse, EverGreen - BigSky Laser Series) capable of double pulsing at 15 Hz was used to illuminate the flow. The laser was operated at the lowest power and highest frequency available. Two concave lenses ($f_l$=-20 cm) were placed in front of the laser to expand the beam size as well as reducing the laser power by spreading the light to ambient. A schematic of the setup is shown in figure 4.2. The light beam horizontally passes through the beam expanding optics hitting the G-2A Nikon cube filter. The cube includes a dichroic mirror, barrier and excitation filters to separate the wavelengths. Then, light vertically transmits though the microscope
objective and illuminates the micro-channel. The filter G-2A set was chosen to match the excitation and emission of the seeding particles (to be explained in section 4.3).
Figure 4.2: Schematic of the micro-PIV setup.
4.3 Tracer particles

The flow was seeded with one micron diameter red fluorescent polystyrene aqueous microspheres (ThermoFisher Scientific). Figure 4.3 shows the fluorescent intensity of the tracer particles and transmittance and reflectivity of the filter set in the present setup, which assist in conducting epifluorescence imaging. The barrier filter blocks all the light wavelengths except the light emitted by the particles.

![Figure 4.3: Excitation and emission of fluorescently labelled particles and transmittance of barrier and excitation filter and dichroic mirror (the data were received from Thermo Fisher and NikonInstruments Co.).](image)

4.4 Calibration

In order to find a calibration constant to convert from pixel space to physical space, a micro-calibration target was used. The calibration constants (optical
magnifications) of the objective lenses are listed in table 4.1. It should be noted
that the strain rate that is the final aim of this chapter is free from the error
of calibration constant as it is possible to calculate it directly from the pixels’
displacements. However, it is necessary for obtaining the velocity in physical
space.

4.5 Micro-PIV experiment

The microfluidic device was horizontally mounted and firmly clamped onto the
inverted microscope stage (explained in section 3.2.4 and shown in figure 3.7).
Prior to securing the microchannel onto the stage, by accurately rotating the
microfluidic device and the camera and using a high-precision $x$-$y$ translation stage,
the channel walls were aligned to the camera sensor. By focusing the camera sensor
on the walls of the microchannel, it was visually confirmed that the microchannel
was oriented to the sensor. However, in section 4.11.3 using data post-processing
methods the exact angle of the misalignment was found and the datasets were
corrected based on the misalignment angle. The maximum misalignment angle
found to be 1.5° degree.

The connection between the microfluidic device and the 1000 µL syringe was
established (explained in section 3.2.4) and using the shut-off valve, the injection
port was blocked. The syringe pump delivered the fluid through the buffer inlet
port(s) at a high flow rate (4000-5000 µL/hr) for about two minutes. Delivering the
fluid to the micro-channels at high flow rates assists in removing the bubbles from
the channels. Then the flow rate was gradually decreased to 50 µL/hr. For each
flow rate 3600 pairs of images were recorded for four minutes (15 Hz). For the next
higher flow rate experiment, the pump was not stopped, rather the flow rate was
increased for the new set of measurements. Stopping the pump may cause bubbles
to form in the microchannel. For each measurement, different time intervals for
laser pulses were chosen. Table 4.2 lists flow rates at which the experiments were
carried out along with the $\Delta t$ of the laser pulses and Reynolds numbers obtained by:

$$Re_{D_h} = \frac{\bar{V}D_h}{\nu}, \quad \bar{V} = \frac{Q}{2wh}, \quad D_h = \frac{2wh}{w+h}$$  \hspace{1cm} (4.1)

where $D_h$ is the hydraulic diameter defined by height ($h$) and width ($w$) of the inlet/outlet channels and $\bar{V}$ is the average inlet velocity. To calculate the average inlet velocity ($\bar{V}$), $\frac{Q}{wh}$ is divided by two, due to the flow bifurcation from the syringe into the two inlets.

<table>
<thead>
<tr>
<th>$Q$ (µL/hr)</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>125</th>
<th>150</th>
<th>175</th>
<th>200</th>
<th>225</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Re_{D_h}$</td>
<td>0.03</td>
<td>0.04</td>
<td>0.06</td>
<td>0.08</td>
<td>0.09</td>
<td>0.11</td>
<td>0.12</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>$\Delta t$ (ms)</td>
<td>12</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$Q$ (µL/hr)</th>
<th>275</th>
<th>300</th>
<th>350</th>
<th>400</th>
<th>450</th>
<th>500</th>
<th>550</th>
<th>600</th>
<th>700</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Re_{D_h}$</td>
<td>0.17</td>
<td>0.19</td>
<td>0.22</td>
<td>0.25</td>
<td>0.28</td>
<td>0.31</td>
<td>0.35</td>
<td>0.38</td>
<td>0.44</td>
</tr>
<tr>
<td>$\Delta t$ (ms)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$Q$ (µL/hr)</th>
<th>800</th>
<th>900</th>
<th>1000</th>
<th>1500</th>
<th>2000</th>
<th>2500</th>
<th>3000</th>
<th>5000</th>
<th>10000</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Re_{D_h}$</td>
<td>0.51</td>
<td>0.57</td>
<td>0.63</td>
<td>0.95</td>
<td>1.26</td>
<td>1.58</td>
<td>1.89</td>
<td>3.2</td>
<td>6.3</td>
</tr>
<tr>
<td>$\Delta t$ (ms)</td>
<td>1.5</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
<td>0.06</td>
</tr>
</tbody>
</table>

### 4.6 Depth of field and depth of correlation in micro-PIV measurements

In macroscopic PIV, depth of field of the PIV measurement or depth correlation is defined by the thickness of the laser light sheet, as the depth of focus of the lens is much larger than the laser light sheet thickness. However, this does not apply to micro-PIV measurements as the depth of field of the objective lens is much smaller than the thickness of the light. The depth of field of a microscope objective is estimated by (Young et al., 1993):
\[
\delta z = \frac{2\lambda_0}{4n_0\left[1 - \sqrt{1 - (NA/n_0)^2}\right]}, \tag{4.2}
\]

where \(\lambda_0\) is the wavelength of the laser light and \(n_0\) is the refractive index between the objective lens and the glass slide (here \(n_0 = 1\) for air). In this experiment the depth of field of the microscope objective is 2.5 \(\mu\)m (table 4.1). However, depth of measurement in micro-PIV is usually larger than depth of field of the microscope objective. The depth of correlation (\(\delta z_c\)) of the microscope objectives are calculated (Olsen and Adrian, 2000) using:

\[
\delta z_c = 2 \left[1 - \sqrt{\epsilon_{th}} \left(\frac{d_p^2}{4\text{NA}^2} + \frac{1.49 (M + 1)^2 \lambda_0}{M^2 \text{NA}^4}\right)\right]^{0.5}, \tag{4.3}
\]

where \(M = 20\times\) and \(\text{NA} = 0.45\) are the magnification and numerical aperture, respectively, of the objective lens. Parameter \(\epsilon_{th}\) is a threshold that determines whether an image of the particle can contribute the correlation function (typically equal to 0.01 (Olsen and Adrian, 2000)). The depth of correlation was 7.4 \(\mu\)m, which is suitable for the experiments in the next chapter as the thickness of the target objects (diameter of the cell of a cyanobacteria filaments) is between 5 to 10 microns.

### 4.7 Pre-processing images

To tackle the common issues in micro-PIV recordings, i.e. low density images with strong background noise, following image processing techniques were applied.

#### 4.7.1 Removing background noise from images

The background noise in the micro-PIV recordings is caused by contaminants, particles adhered to the glass slide and microchannel walls or the ambient light. In
order to remove the background noise involved, ensemble-averaging of 100 images in the first exposure and 100 images in the second exposure was applied. This was followed by subtracting the former from each image in the first exposure and the latter from each image in the second exposure. Figures 4.4a and 4.4b show an example of an image before and after background removal. An arbitrary interrogation window was selected from the same position (red rectangle) in figures 4.4a and 4.4b and they are shown in figures 4.5a and 4.5b. The effect of background removal on the correlation peak is explained in section 4.8.

Figure 4.4: An example of the cropped micro-PIV recordings of the central region of the cross-slot junction in the first laser exposure. The red rectangle represents a $32 \times 32$ interrogation window zoomed in figure 4.5.
4.7.2 Overlaying of low image density (LID) images (first step)

In micro-PIV, seeding density is minimised to reduce the noise produced by the out-of-focus particles. The concentration of particles in an image pair of micro-PIV images is usually enough for particle tracking velocimetry (PTV). In PTV, each velocity data point is obtained by the movement of each particle. Therefore, to obtain enough velocity vectors in the region of interest interpolation (that increase the error) is required. As we are interested in calculating the strain rate that is the derivative of velocity, a larger number of velocity vectors is required, and therefore PTV is not a suitable option.

When the seeding density is too low, the standard cross correlation methods fail to produce sufficient signals to obtain accurate and reliable results. To tackle the low image density (LID) issue, digital image processing techniques were applied to artificially increase the number of particles per image. There are three common techniques to alleviate this problem: “Average Image Method”, “Average Velocity Method” and “Average Correlation Method”. Using the Average Velocity Method is usually not recommended as all the instantaneous velocity vectors must be valid before averaging the velocity vectors, otherwise the results will be erroneous (Meinhart et al., 2000b). This method is only applied when instantaneous velocity vectors have physical importance. The Average Image Method is limited for increasing the accuracy. This limitation stems from the fact that if too many images are added together the visibility of the particles is diminished. The Average Correlation Method is known as the superior method compared to the other methods (Wereley et al., 2002). It is however, not possible to study time-resolved data when this method is employed. In this chapter a combination the Average Image Method and the Average Correlation Method was used.

The averaging techniques are usually applied in micro-PIV experiments because the Reynolds numbers in the microchannels are very low and the flow can be
considered laminar and steady or periodic. However, we are also interested in studying the steadiness of the position of the stagnation point. This enables us to investigate the stagnation point fluctuations issue that is experienced in the miniature four-roll mill (Appendix A).

After investigating the different methods, the following procedure was found to be most suitable. Firstly, the Average Image Method was applied, i.e. overlaying of six LID-PIV images with 50% overlap to satisfy Nyquist criterion (table 4.1). The algorithm is graphically illustrated in figure 4.6. This enables us to reach approximately 9.5 particles per interrogation window on average (ideal for PIV measurements), compared to about only three particles per interrogation window before the ensemble averaging. Figures 4.4 and 4.5 show increases in the numbers of particles per image and interrogation window by comparing figure 4.4c and 4.5c.
to 4.4b and 4.5b, respectively. The effect of ensemble-averaging on the correlation peak is explained in section 4.8.

The above procedure allows for the possibility of studying the time-resolved velocity field. However, instead of each instantaneous velocity field we have time-averaged results over 0.45 ms.

### 4.8 Processing methods

Using a code developed by the Fluid Mechanics Group at the University of Melbourne, the images were correlated and processed. Some of the methods and algorithms that were used are briefly described here. In PIV measurements the cross-correlation coefficient map can be written as:

\[
RR(\delta x, \delta y) = \frac{\sum_{j=1}^{N_H} \sum_{i=1}^{N_W} I_A(x_i, y_j)I_B(x_i + \delta x, y_j + \delta y)}{\sigma_{I_A}\sigma_{I_B}},
\]

where \(I_A\) and \(I_B\) are the distributions of the particle intensities within interrogation windows in the first and second exposure, respectively. The standard deviation is represented by \(\sigma\). The normalised correlation is carried out in Fourier space using the Fast Fourier Transform (FFT) and then the correlation map is transformed back to pixel/physical space (Lewis, 1995).

The local displacement vector for an interrogation window is equal to the distance between the correlation map centre and the location of the correlation peak in pixels (e.g. figure 4.7a).
4.8.1 Interrogation strategy

If the number of the particles within an interrogation window is optimised, bias and random estimation errors are reduced. To do this, a multigrid algorithm with adaptive central difference interrogation (CDI) offsetting was employed, in which the interrogation window size is successively reduced to the desired resolution (the parameters are listed in table 4.1). The multigrid technique was combined with window deformation algorithm (introduced by Huang et al. (1993a,b)) to reduce the random error caused by deformation of the measured flow.

4.8.2 Ensemble correlation technique (second step)

After applying the first averaging method and correlating the images, the Average Correlation Method was applied to obtain a more accurate velocity field and the strain rate. In this technique the correlations were averaged over \( N > 500 \) realisations. As the measurements were carried out over four minutes, after applying the Average Image Method, \( N=1792 \) realisations are available. However, averaging correlations over the first 500 realisations was sufficient for the convergence of the correlation map.

4.8.3 Sub-pixel accuracy

To reach sub-pixel accuracy (to avoid pixel locking), a “Gaussian fit” estimator (that yields the best results amongst other three-point estimators, such as “Centroid” and “Parabolic fit”) was fitted to the correlation peak to minimise pixel locking (Adrian and Westerweel, 2011).
4.8.4 Correlation peak detection

As previously mentioned the local displacement obtained from each interrogation window is determined by the location of the correlation peak. Therefore, an increase in probability of the correlation peak detection leads to a decrease in the probability unrealistic/spurious velocity existence. To determine the probability of the detection of the tallest peak, “peak detectability” \( (D_0) \) is defined as the ratio of the amplitude of the tallest peak to the second tallest peak (Coupland and Halliwell, 1988):

\[
D_0 \equiv \frac{\text{Amplitude of the tallest peak in correlation function}}{\text{Amplitude of the second tallest peak in correlation function}}.
\] (4.5)

To compare the effect of the pre-processing techniques (section 4.7) and averaging methods (section 4.8.2) on the detectability and signal-to-noise ratio, the correlation map of an arbitrary interrogation window is plotted in figure 4.7. In figure 4.7a the detectability \( (D_0) \) is about one, which means the probability of detecting the correlation function is zero. Upon applying the background removal method, the level of noise was decreased, however, still a very low level of signal-to-noise ratio can be seen in figure 4.7b. Once the Average Image Method was employed the detectability increased to about three times its initial value that yields valid data (figure 4.7c). However, it should be noted that this was an example of an interrogation window in some of the interrogation windows the detectability was still too low to produce good results. This issue is investigated in section 4.9. Finally, by employing the Average Correlation Method over 500 realisations, an ideal correlation map with a distinct peak \( (D_0 \approx 50) \) was obtained (figure 4.7d).
4.9 Spurious vector detection and treatment

The normalised median test, proposed by Westerweel and Scarano (2005), was used to detect outlier data. In this method velocity vectors are identified as outliers if there is a large deviation with respect to the adjacent data points. Here, the acceptable noise level and the data point neighbourhood radius were set to 0.1 and 1 pixel, respectively. In the case of using the Average Image Method for each semi-instantaneous datum, the normalised median test was employed to detect the spurious vectors, then they were corrected using second order correlation (Wereley and Meinhart, 2001).
Figure 4.8: Valid and spurious local displacement vectors in pixels over the central region of 500 × 500 pixels.

Figure 4.8 shows the effect of the averaging methods on the number of detected spurious vectors. On average, more than 30% of vectors were labelled as invalid data when no averaging technique was used. Figure 4.8a is an example of the displacement vector field for this case. Once the six LID-PIV recordings were overlaid the number of invalid vectors markedly decreased to less than 8% on average (figure 4.8b – before any spurious correction). By replacing the invalid
data (figure 4.8c) the number of spurious velocity data points reduced to less than 0.05% and the displacement vector fields became very smooth, similar to a linear velocity field flow. Finally, after applying the Average Correlation Method no spurious vectors were detected (figure 4.8d).

### 4.10 Uncertainty and errors due to Brownian motion

Brownian motion of sub-micron size particles is one of the main sources of errors in micro-PIV measurements. The error due to the Brownian motion is estimated by (Santiago et al., 1998):

\[ \epsilon_B \approx \frac{1}{u} \sqrt{\frac{2D}{\Delta t}}, \]  

(4.6)

where \( u \) is the characteristic velocity (here local velocity in \( x \)-direction) and \( D \) is the diffusion coefficient of microspheres that can be estimated by (Einstein, 1906):

\[ D = \frac{K_B T}{3\pi \mu d_p}, \]  

(4.7)

where \( K_B = 1.38924 \times 10^{-23} \) J/K is the Boltzmann’s constant, \( T \) is the absolute temperature, \( \mu \) is the dynamic viscosity of the fluid and \( d_p \) is the diameter of a particle.

Due to the relatively high speed flow (order of 1000 \( \mu \)m/s) (Meinhart et al., 1999), the maximum relative error associated with the Brownian motion was insignificant (less than maximum 2% in figure 4.9). The 2% error corresponds to the the region very close (8 \( \mu \)m) to the stagnation point, by increasing the distance from the centre this error significantly reduces. Moreover, figure 4.9 shows the errors for
the lowest flow rate \( Q = 50 \, \mu\text{L/hr} \), which has the highest amount of errors due to the lowest velocity. For higher flow rates this error is markedly less. This amount is considerably lower than that of other micro-PIV experiments, which is usually about 10% \( (\text{Meinhart et al., 1999, 2000a; Santiago et al., 1998}) \).

\[
\begin{align*}
\delta x_{\text{uncertainty}} &= \frac{d_e}{10M}, \\
(4.8) \\
\end{align*}
\]

where \( d_e \) is the effective particle diameter image, which can be estimated by \( (\text{Adrian, 1991}) \):

\[
\begin{align*}
d_e &= M(d_p^2 + (1.22\lambda_0/\text{NA})^2)^{1/2}. \\
(4.9) 
\end{align*}
\]

As stated in Prasad et al. (1992), if the diameter of a particle in an image is resolved within three to four pixels, the location of the particle image correlation peak can be obtained within 10% of the particle diameter in the image. This means that the measurement uncertainty can be determined by:

\[
\begin{align*}
\text{Figure 4.9: Relative error due to the Brownian motion versus local velocity at } Q = 50 \, \mu\text{L/hr}. 
\end{align*}
\]
In our experiments the effective diameter of the particle image was \( d_e = 28.8 \mu m \) and when it is projected back to the flow it is 1.44 \( \mu m \). Therefore, using equation 4.8 the micro-PIV measurement uncertainty was 144 nm.

### 4.11 Results and discussions

In this section the post-processed data (velocity field and strain rate) is presented and compared with the pure straining flow that has a linear velocity field \( (u = \gamma x \) and \( v = -\gamma y \), where \( \gamma \) is the constant strain rate).

#### 4.11.1 Velocity field

Figure 4.10a shows an example of the ensemble-averaged velocity vector map and streamlines in the 300 \( \mu m \times 300 \mu m \) central region of the cross-slot junction (previously shown in figure 2.7) at the flow rate of 75 \( \mu L/hr \). To qualitatively compare the flow to the pure straining flow, the velocity magnitude contour is depicted in figure 4.10b. The circular regions in this figure confirm the existence of the linear velocity field, as pure straining flow has a circular velocity magnitude profile \( (|u| = \gamma \sqrt{x^2 + y^2}) \).

#### 4.11.2 Deviation of measured velocity field and strain rate from the ideal linear velocity field

To calculate the strain rate, the gradients of velocity map \( \left( \frac{\partial u}{\partial x} \right) \) and \( \left( \frac{\partial v}{\partial y} \right) \) were calculated by fitting a Savitzky-Golay differentiation filter to a set of 5 \( \times \) 5 velocity data points to a third-order polynomial in the least-squares sense. Then strain rate was calculated by:
In order to quantitatively compare the measured velocity field and the uniformity of the strain rate to an ideal hyperbolic flow, equations:

\[ u = \gamma_{eq}(x - X_o), \quad v = -\gamma_{eq}(y - Y_o), \quad (4.11) \]

that present a linear velocity field with an unknown centre of \((X_o, Y_o)\) and equivalent strain rate \(\gamma_{eq}\) were fitted to the velocity field with a least-square error approximation. For each velocity vector, two equations were available. Using the strain rate that was obtained from the gradient of velocity (given by equation 4.10), these equations could be converted to linear equations. Therefore, linear equations were solved, and \(X_o\) and \(Y_o\) were obtained.
However, in order to obtain more accurate results, apart from $X_o$, $Y_o$ the equivalent strain rate ($\gamma_{eq}$) is assumed to be unknown, thereby having a set of non-linear equations. To solve the new set of non-linear equations, $X_o$ and $Y_o$ that previously calculated, were used as initial conditions to solve the non-linear equations. Subsequently, non-linear equations were solved using the Newton-Raphson method, and the position of centre ($X_o$, $Y_o$) and the equivalent strain rate ($\gamma_{eq}$) were determined.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.11.png}
\caption{The solid lines represent the least square fits to the ensemble-averaged velocity and the error bars correspond to twice the standard deviation of the 95\% confidence level.}
\end{figure}

The black and red solid lines in figure 4.11 show the least square fits to the ensemble-averaged values of $u$ and $v$, respectively. The error bars of the $u$-velocity and $v$-velocity data show twice the standard deviation of the 95\% level along the $y$-axis (extensional axis) and $x$-axis (compressional axis), respectively. With the increase in the distance from the stagnation point, an increase in deviation for both velocity components from the linear profile can be seen. This deviation might be rooted in the effect of the side walls. More importantly, it is evident that the error
bars are not symmetric around the $x$ and $y$-axes. This means bias errors exist which are not caused by the effect of the cross-slot walls, as the channel has a symmetrical geometry.

4.11.3 Removing the bias error due to camera misalignment

To provide more information regarding the increase in the deviation from the linear velocity field, variations of $u$ at three different $x=$constant locations and the variation of $v$ at three different $y=$constant locations are plotted in figure 4.12. To investigate the velocity profile of the various flow rates, non-dimensional parameters are defined as:

$$x^* = \frac{x}{w}, \quad y^* = \frac{y}{w},$$

(4.12)

$$u^* = \frac{u}{\gamma w}, \quad v^* = \frac{v}{\gamma w}.$$  

(4.13)

From the data provided in figure 4.12, it can be seen that $u$ and $v$ for all flow rates collapsed at each location, however, the velocity profiles at different locations vary differently from the linear expected trend. The velocity field at the locations closer to the centrelines of the channels shows more linear behaviour. A closer look at the $x^* = 0$ or $y^* = 0$ locations shows a discrepancy between the slope of the measured velocity and the ideal linear velocity (given by equation 4.11). This suggests an angular misalignment of the camera and the microchannel. To address this problem, the angle between $x^* = y^*$ and $u^*(x^* = 0)$ was determined with the maximum misalignment angle of $1.5^\circ$ degree. Based on this misalignment angle the whole velocity field was tilted around the intersection of these two lines.
Figure 4.12: Comparison of the measured $u$ and $v$ velocity components at different $x^* =$ constant and $y^* =$ constant locations with ideal linear velocity field, at different flow rates.

To study the effect of this rotation, the initial values of standard deviations of the $u^*$ and $v^*$ and their values after rotation are represented in figure 4.13. It should be noted that figure 4.13 shows the same values as the error bars in figure 4.11. Therefore, the bias error in the deviation of the measured velocity from the ideal linear flow was removed from the data and the deviations reduced to half of their
(a) The standard deviations of $u^\ast$. 
(b) The standard deviations of $v^\ast$.

**Figure 4.13:** The standard deviations of $u^\ast$ and $v^\ast$ were reduced to half of their initial values by tilting the velocity field and removing the bias error due to the camera misalignment.

initial values. It can be also concluded that for all the flow rates, the deviation from a uniform straining flow in the central region ($-0.6w < x, y < 0.6w$) was less than 2 %. In other words, in this region the relation $\frac{du}{dx} = \frac{dv}{dy} = \gamma$ is held with the accuracy of 2 %.

### 4.11.4 Fluctuation of the stagnation point

As previously mentioned we obtained the time resolved velocity to investigate the possible variation of the stagnation point location with time and compared this with our miniature four-roll mill (in Appendix A). Figure 4.14 illustrates the fluctuation of the stagnation point and $\gamma_{eq}$ for the case of flow rate $Q = 700 \, \mu L/hr$. The standard deviation of the stagnation point variation in $x$ and $y$ directions are 0.50 $\mu m$ and 0.63 $\mu m$, respectively. Using the same method it is confirmed that these values were less than one micron for all the flow rates, whereas those of the four-roll mill were higher than 20 $\mu m$. This result confirms the possibility of the trapping and confining of a target object larger than one micron at the zero-velocity point.
It should be noted that there is an observable periodic behaviour in $\gamma_{eq}$ data (figure 4.14), whereas no such periodic feature is observed in the location of the stagnation point. We suspect that the fluctuation in $\gamma_{eq}$ is due to the pumping of the fluid and related to the flow rate. If this is true, we would expect the dominant frequency in strain rate ($f_\gamma$) scales linearly with flow rate ($Q$). A Fast Fourier Transform (FFT) of $\gamma_{eq}$ in figure 4.14 shows $f_\gamma \approx 0.22$ Hz. After analysing time-series for all flow rates ($Q$), we found that indeed $f_\gamma$ has a linear relationship to $Q$ ($f_\gamma = 1.13Q$).

In any case, the amplitude of this frequency is so small that it hardly affects the uniformity of the flow strain rate (The standard deviation of 2%. However, $X_o$ and $Y_o$ did not show this trend, most likely because the inlets are symmetric and the oscillation cancels out.

![Graph showing fluctuations](image)

**Figure 4.14**: Fluctuation of the stagnation point and the strain rate over the four minute measurement. The variation of the stagnation point was less than 1 µm for all the flow rates and the variation of the strain rate was less than 4%. 
4.11.5 Strain rate at the cross-slot region

To provide the possibility of the investigation of the uniformity of the strain rate for all the flow rates at the same time, the non-dimensional parameter $\gamma^*$ is defined as:

$$\gamma^* = \frac{\gamma - \gamma_{eq}}{V/(2w)}, \quad (4.14)$$

Probability Density Functions (PDF) of the non-dimensional strain rate for all of the flow rates are shown in figure 4.15. The figure shows the same standard deviation in the central region for all flow rates. The relative standard deviation was about 4%.

Figure 4.15: PDF of non-dimensional parameter $\gamma^*$ for various flow rates, showing the same deviation in the central region of the cross junction.
Finally the strain rate of the velocity field at a central region of the cross-slot microchannel \((-0.6w < x, y < 0.6w)\) is plotted in figure 4.16. The error bars represent twice the standard deviation of the strain rate over this region. The strain rate and flow rate are found to be related by a linear relationship with equation:

\[
\gamma = 0.0493Q + 0.0426, \tag{4.15}
\]

where \(Q\) is in (\(\mu\)L/hr) and \(\gamma\) is in (s\(^{-1}\)). The strain rate in the cross junction can be crudely estimated using (Ulloa et al., 2014):

\[
\gamma_{est} = \left(\frac{Q}{hw}\right) \left(\frac{1}{w}\right), \tag{4.16}
\]

which is the average inlet velocity divided by half of the width of the channel, where velocity reaches from its maximum value to zero. Therefore, due to the effect of the wall this formula underestimates the strain rate. However, it reasonably agrees with the experimentally determined strain rate. This estimation is shown in figure 4.16 along with the measured strain rates.

By comparing the obtained strain rate to those of previous studies in a four-roll mill, it is shown that strain rates of up to 142 s\(^{-1}\) could be reached in this microfluidic device, whereas 6 s\(^{-1}\) is the highest reported strain rate in conventional four-roll mills (Andreotti et al., 2001). Therefore, not only does the cross-slot microchannel enable the study of micron-sized objects in a uniform straining flow, but it also provides the possibility of applying a high strain rate to micron-scaled object.

It should be noted that due to the no-slip condition at the top and bottom walls of the cross-slot channel, the flow is approximately two-dimensional and the strain rate varies at different \(z\)-planes. The strain rates reported in this study correspond
to the average strain rate along $\delta z_c \approx 7 \, \mu m$ over the mid-section of the channel, which is close to the thickness of *A. circinalis* filaments (about 5 to 10 $\mu m$). Therefore, when a *A. circinalis* filament is held at the stagnation point in the mid-section of the channel, it will be exposed to a flow with the strain rates that are measured in this chapter (figure 4.16).

Considering the laminar flow, it is possible to estimate this variation by assuming a parabolic velocity profile in the x-z or y-z plane. By this assumption it is found that the strain rate changes within $\pm 4\%$ in the z-direction within the depth of measurement.
4.12 Summary

The experimental procedure, data processing and the results of micro-PIV measurements in a cross-slot type microfluidic device were described. Using a combination of the common averaging methods instead of a single ensemble-averaging method enabled us to study the fluctuation of the stagnation point and compare it with the stagnation point fluctuation problem that was encountered in our miniature four-roll mill (Appendix A). The results show that the variation of the stagnation point was less than one micron for all the flow rates. This confirms the possibility of trapping objects larger than one micron in the straining flow in the cross-slot microchannel.

By fitting an ideal stagnation point velocity field equation to the measured velocity field, the bias errors in the deviation of the micro-PIV results from ideal linear flow, due to the misalignment of the camera were removed. This error was mentioned by Wereley and Gui (2001, 2003) in previous works, however no correction has been carried out. The post-processed results illustrate deviation of only 2% from ideal hyperbolic flow for all of the flow rates at the central region of \((0.6w \times 0.6w)\).

The strain rate in flow at various flow rates shows existence of a linear relation between these two parameters. More importantly, the results confirm reaching a strain rate of up to 142 \(s^{-1}\) at a high flow rate, whereas 6 \(s^{-1}\) was the highest reported strain rate reported in four-roll mills (Andreotti et al., 2001).
Chapter 5

Exposing microorganisms to straining flow using active control

In this chapter we present the use of a microfluidic trap to confine waterborne microorganisms and expose them to long-term straining flows. As explained in chapter 2, we focus on Dolichospermum circinale (formerly Anabaena circinalis (Wacklin et al., 2009)), of the cyanobacteria species, which is a cause of significant water contamination worldwide (Cook et al., 2010).

In the preceding chapter the flow in the cross-slot type microfluidic was quantified at different flow rates, thereby quantifying the strain rates that microorganisms would experience if they are trapped at the centre of the cross-junction.

The implementation of the microfluidic trap, which will be equipping the cross-slot microchannel with an active feedback control will be thoroughly described. Prior to the implementation of the microfluidic trap and conducting the experiments, the control system was simulated, and the possibility of trapping objects at different conditions was investigated.
5.1 Basic concepts of the hydro-microfluidic trap

The functioning of the microfluidic trap is based on the active flow manipulation to confine objects at the stagnation point of the cross-junction.

5.1.1 Semi-stable equilibrium point in the straining stagnation point flow

In the preceding chapter, we found that there exists a reasonably constant strain rate flow field in the central region of the cross-junction (0.6 of the width of the cross-region schematically shown by the red square in figure 5.1).

\[
\phi = \frac{\gamma}{2}(x^2 - y^2),
\]

which represents a saddle surface and a saddle point (at \(x = 0\) and \(y = 0\)) in the domain of the potential function (the circle in figure 5.1). In principle, the zero value points of the potential function of a system show the critical points of the system. To investigate the stability of the critical points the derivative of the potential
function is required. The derivative of the saddle surface along the extensional axis is negative, whereas along the compressional axis it is positive. Therefore, the potential function of the velocity has a semi-stable equilibrium point (shown in figure 5.2). In other words, the stagnation point is a stable equilibrium point along the compressional axis (inlet channels), and an unstable equilibrium point along the extensional axis (outlet channels). This means if an object is placed at the zero-velocity (stagnation) point, it will finally be displaced by an external disturbance/fluctuation. Also in the case of sub-micron objects, Brownian motion plays a crucial role in displacing the objects from the stagnation point (Tanyeri and Schroeder, 2013). Therefore, in this thesis, a flow-based technique introduced by Tanyeri et al. (2010), was employed to confine microorganisms at the stagnation point, thereby exposing them to long-term straining flow. This method is an image-based control technique in which the stagnation point is actively repositioned to push a target object towards the centre of the cross-slot channel/centre of the trap (the hollow cross in figure 5.1).

5.1.2 Continuous stagnation point repositioning

If the two outlets of the cross-channel have the same flow resistance (to be defined in section 5.2.2), the inlet streams are equally bifurcated to the two outlets (shown in figure 5.1). If there is a constraint on one of the outlets (e.g. the constraint shown at the bottom outlet in figure 5.3a), the outlet flow rates will be no longer equal because of the inequality in the flow resistance of the outlets. Moreover, the stagnation point is displaced towards the outlet with higher flow resistance. Therefore, if the cross-section area of the outlets constantly varies, the stagnation point will be continuously repositioned along the extensional axis.

In this work, repositioning the stagnation point (shown in figure 5.3b) is conducted by implementing a fixed width constriction in one of the outlets, and a variable height constriction on the other (Tanyeri et al., 2010). The former is shown in
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(a) The hyperbolic streamlines in the cross-junction of the microfluidic trap.  

(b) The potential function (saddle surface) in the microfluidic trap (shown in figure 5.2a) and the semi-stable equilibrium stagnation point (bottom figure) Velocity vector map of the pure straining flow (top figure).

**Figure 5.2:** The Potential function of velocity field in the cross-slot microchannel and semi-stable equilibrium point along extensional axis (Tanyeri et al., 2010).

the bottom outlet and the latter is shown in red on the top outlet in figure 5.3a. Figure 5.3c shows the section A-A of the variable height constriction, known as an on-chip membrane valve (in figure 5.3a), and how its deformation constricts the height of the fluidic channel. The control channel is filled with pressurised gas, which results in the deflection of the thin membrane, thereby constricting the height of the fluidic channel. It is called the variable constriction as changes in the gas pressure lead to changes in the deflection of the membrane, and consequently constricting the channel at different heights.
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5.1.3 Automation of the stagnation point repositioning to confine a target object at the channel centre

In the microfluidic trap, when a target object enters the cross-slot region (shown in figure 5.4a), a camera captures and streams the image of the cross-junction to a
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Figure 5.4: The effect of displacing the stagnation point on the flow streamlines and the trajectory of an object in cross-junction. The red ellipse, solid black circle, hollow circle and the hollow cross represent the target object, its centroid, the stagnation point and the centre of the channel, respectively.

(a) An object (shown by the red ellipse) entering the cross-slot region. The control camera streams the image of the cross-region to a computer to detect the object and find its centroid.

(b) Once the centroid of the object in figure 5.4a is determined, the control layer is pressurised and the stagnation point and streamlines are displaced, resulted in forcing the object to move towards channel centre.

(c) After the target was forced to move towards the centre of the cross-junction, by changing the pressure in the control channel the stagnation point moves towards the centre, too. Eventually, both the stagnation point and the target converged at the centre.

A computer. Using image processing methods the shape of the object is determined and the position of the object centroid is computed (to be described in section 5.3.3). Based on the location of the object in extensional direction (y-axis), the ratio of the outlet flow rates is changed and consequently the stagnation point is repositioned. The repositioning is carried out using the on-chip membrane valve, which allows the flow streamlines to be manipulated. This manipulation places the target object between the stagnation point and the centre of the channel (shown in figure 5.4b), thereby exerting a hydrodynamic force on the target object towards the trap centre. The manipulation results in placing the object on a new streamline and moving the object towards the channel centre. All of these steps are repeated until both the target and the stagnation point converge in the centre of the trap/channel (shown in figure 5.4c), and at this stage the pressure stays unchanged. However, if due to an external or internal disturbance the target object is displaced from the centre, the same procedure is re-applied forcing the
object to return to the centre.

5.1.3.1 Feedback Control algorithm

A linear feedback control algorithm was implemented to update the pressure in the control layer and displace the stagnation point using (Tanyeri et al., 2010):

$$P_{\text{val}} = P_{\text{tc}} + K_P K_C e_{\text{tt}},$$  \hspace{1cm} (5.2)

where $P_{\text{val}}$ is the pressure in the control channel (on-chip valve), $P_{\text{tc}}$ is the required pressure to keep the stagnation point at the trap centre. The proportional gain is presented by $K_P$ and it can be considered as a constant ($K_P = -1.5$) and $K_C$ is a factor to convert pressure to distance. If there is a linear relation between the pressure and the stagnation point position, $K_C$ will be a constant. The offset error ($e_{\text{tt}}$) in equation 5.2 is defined as:

$$e_{\text{tt}} = Y_{\text{tp}} - Y_{\text{tc}},$$  \hspace{1cm} (5.3)

which is the distance between the position of target ($Y_{\text{tp}}$) and trap centre ($Y_{\text{tc}}$) along the extensional axis (shown in figure 5.4a). Figure 5.5 depicts the flowchart of the steps employed for confining a target object in the centre of the cross-slot junction.
Figure 5.5: Algorithm for confining a target object in the cross-slot region of the microchannel, which is schematically shown in figure 5.4.

5.2 Simulation of the control system to trap a target object

The effect of the length, width and height of the constraints on the outlet channels on the ratio of the outlet flow rates and the position of the stagnation point can be studied using the analytical solution of the Navier-Stokes equations in rectangular channels. Here we adopt the parametric study carried out by Tanyeri et al. (2011a) to simulate the microfluidic trap and its control system. The simulation of the control system assists in predicting trajectory of a target object and ascertaining
the possibility of the trapping at different conditions such as flow rate, initial position of the target and the trap time response.

Table 5.1 lists the dimensions and normalised parameters of the microfluidic device (fabricated in chapter 3) used to simulate the control system.

Table 5.1: Specifications of the channel and its constraints and normalised parameters used in the simulation.

<table>
<thead>
<tr>
<th>Channel and constraints (µm)</th>
<th>Normalised dimensions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel width ($w$)</td>
<td>400</td>
</tr>
<tr>
<td>Channel height ($h$)</td>
<td>40</td>
</tr>
<tr>
<td>Outlet channel length ($L$)</td>
<td>7000</td>
</tr>
<tr>
<td>Valve height ($h_v$)</td>
<td>(variable)</td>
</tr>
<tr>
<td>Valve length ($l_v$)</td>
<td>400</td>
</tr>
<tr>
<td>Fixed constriction width ($w_c$)</td>
<td>100</td>
</tr>
<tr>
<td>Fixed constriction length ($l_c$)</td>
<td>1800</td>
</tr>
</tbody>
</table>

$w_v^* = h_v/h$ (variable)

$l_v^* = l_v/L$ 0.0571

$w_c^* = w_c/w$ 0.25

$l_c^* = l_c/L$ 0.2571

5.2.1 Laminar flow in a rectangular microchannel

The Navier-Stokes equations and boundary conditions for a rectangular channel with the height of $h$, width of $w$ and length of $L$ (with an aspect ratio of $\alpha_r = h/w$ and in our case $\alpha_r = 0.1$), are given by (Bruus, 2008):

$$
\left( \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2} \right) u = -\frac{\Delta P}{\nu L},
$$

where $-\frac{w}{2} \leq y \leq \frac{w}{2}$ and $0 \leq z \leq h$,

(5.4)

Boundary Conditions: $u = 0$ at

$$
\begin{cases} 
  y = \pm\frac{w}{2} \\
  z = 0 \quad \text{and} \quad z = h 
\end{cases}
$$

where the stream-wise velocity component $u(y, z)$ is a function of $y$, $z$ variables. Axes of the coordinate along the stream-wise (length), span-wise (width) and
transverse (height) of the rectangular channel are represented by $x$, $y$ and $z$, respectively. The flow was assumed to be pressure-driven ($\Delta P$ is the pressure difference over the distance $L$ between the channel inlet and outlet), steady state and fully developed with no slip conditions on the walls. The analytical solution of equation 5.4 is provided by a Fourier series expansion (Bruus, 2008):

$$u(y, z) = \frac{4h^2\Delta P}{\pi^3\nu L} \sum_{n,\text{odd}} \frac{1}{n^3} \left[ 1 - \frac{\text{cosh}(n\pi \frac{y}{h})}{\text{cosh}(n\pi \frac{w}{2h})} \right] \sin\left(\frac{n\pi z}{h}\right). \quad (5.5)$$

By integrating the velocity (equation 5.5) over the rectangular cross-section area, the volumetric flow rate can be expressed as (Bruus, 2008):

$$Q = 2\int_0^{w/2} dy \int_0^h u(y, z) dz = \frac{h^4\Delta P}{12\nu L\alpha} \left[ 1 - \sum_{n,\text{odd}} \frac{192\alpha}{(n\pi)^3} \tanh\left(\frac{n\pi}{2\alpha}\right) \right]. \quad (5.6)$$

### 5.2.2 Flow resistance of the outlets of the cross-slot channel

Based on the analogy with Ohm’s law, the resistance of the laminar flow in a channel is defined as (Akers et al., 2006):

$$R = \frac{\Delta P}{Q}. \quad (5.7)$$

Therefore, in the cross-junction shown in figure 5.6, the ratio of the outlet flow rate with a fixed width constriction ($Q_c$) to the total flow rate ($Q = Q_n + Q_c$) can be stated as:

$$\frac{Q_c}{Q} = \frac{\Delta P_c}{\Delta P_c + \Delta P_n} = \frac{1}{\frac{R_n}{R_c} + \frac{1}{R_n}} = \frac{R_n}{R_n + R_c} = \frac{1}{1 + \frac{R_c}{R_n}}, \quad (5.8)$$
Figure 5.6: Schematic of the cross-slot junction with a fixed width constriction on the right-hand side outlet, and no constriction on left-hand side outlet. The width and the length of the constriction are shown by $w_c$ and $l_c$, respectively. The height of the constriction is the same as the channel height $(h)$. The outgoing flow rate from the constricted channel and its corresponding resistance are represented by $Q_c$ and $R_c$, respectively. Those of the channel with no constriction are shown by $Q_n$ and $R_n$, respectively.

where $R_n$ and $R_c$ are the flow resistance of the channel with no constriction and with a fixed width constriction, respectively. Also it is assumed that the pressure drop in both outlets is the same ($\Delta P_c = \Delta P_n$). This assumption is correct if the length of the constriction is much smaller than the outlet length ($l_c << L$).

5.2.3 The effect of the constriction width and length on the ratio of the flow rates through the outlet channels

Using equations 5.6 and 5.7, the resistance of the outlet channel with no constriction ($R_n$ shown in the left outlet in figure 5.6) can be expressed as:

$$R_n = \frac{12 \nu L \alpha}{h^4} \left[ 1 - \sum_{n, \text{odd}}^{\infty} \frac{192 \alpha}{(n\pi)^5} \tanh \left( \frac{n\pi}{2\alpha} \right) \right]^{-1}.$$  \hspace{1cm} (5.9)

If there is a constriction with the length and width of $l_c$ and $w_c$, respectively, and the same channel height $(h)$, the resistance of the whole constricted outlet channel
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(the right outlet with the length of $L$ in figure 5.6) using $\Delta P_c = \Delta P_n(1-l'_c) + \Delta P_{ctr}$ is given by:

\[
R_c = R_n(1 - l'_c) + R_{ctr}, \tag{5.10}
\]

where the resistance of the constriction part is represented by $R_{ctr}$ (the part of the right outlet, that has the length of $l_c$) and $l'_c$ is the normalised constriction length (listed in table 5.1). Using equations 5.6 and 5.7, $R_{ctr}$ can be stated as:

\[
R_{ctr} = \frac{12\nu L\alpha}{h^4} \left( \frac{l'_c}{w'_c} \right) \left[ 1 - \sum_{n,\text{odd}}^{\infty} \frac{192}{(n\pi)^5} \left( \frac{\alpha}{w'_c} \right) \tanh \left( \frac{n\pi w'_c}{2\alpha} \right) \right]^{-1}, \tag{5.11}
\]

where $w'_c$ is the normalised constriction width (listed in table 5.1).

Using equation 5.8 – 5.11, the variation of the relative flow rate in the constricted outlet channel versus variation of the length and width of the fixed constriction is shown in figure 5.7. The marked point is the specification of the channel listed in table 5.1.

![Figure 5.7](image)

**Figure 5.7:** The effect of the normalised length ($l'_c$) and width ($w'_c$) of the fixed width constraint (in figure 5.6) on the relative flow rates.
5.2.4 The effect of the constriction height (membrane valve) on the ratio of the flow rates through the outlet channels

The same analogy for calculating the resistance of the outlet with the fixed width constriction is applied to compute the resistance of the outlet with variable height constriction ($R_v$) that is shown in figure 5.8. Therefore, $R_v$ is given by:

$$R_v = R_n(1 - l_v^*) + R_{val}, \quad (5.12)$$

where $R_{val}$ is the resistance of the constrictor section with the length of $l_v$ and height of $h_v$ (the red feature in figure 5.8). Using equations 5.6 and 5.7, $R_{val}$ is given by:
\[ R_{val} = \frac{12\nu L\alpha}{h^4} \left( \frac{l_v^*}{h_v^3} \right) \left[ 1 - \sum_{n,odd}^\infty \frac{192\alpha h_v^*}{(n\pi)^5} \tanh \left( \frac{n\pi}{2\alpha h_v^*} \right) \right]^{-1}, \quad (5.13) \]

where, \( h_v^* \), \( l_v^* \) are normalised constriction height and length (listed in table 5.1), respectively.

Therefore, in the cross-junction shown in figure 5.8, (using the similar procedure to derive equation 5.8) the ratio of the outlet flow rate with a variable constriction \( Q_v \) to the total flow rate \( Q = Q_v + Q_c \) can be stated as:

\[ \frac{Q_v}{Q} = \frac{1}{1 + \frac{R_v}{R_c}}. \quad (5.14) \]

Using equations 5.10-5.14 the effect of the variable height constriction on the relative flow rate with and without the fixed width constriction (on the other outlet) is shown in figure 5.9. Comparing these two curves, it is confirmed that the existence of both fixed and variable constraints on both outlets resulted in obtaining a wider range of flow rate variation (compared to the case that there is only variable height constraint). Achieving a wider range of flow rate variation enables repositioning of the stagnation point in the wider range. In other words, the fixed width constraint works as an offset for the ratio of the flow rate and the position of stagnation point in the cross-junction. Therefore, an initial pressure in the control channel is required to deflect the membrane and equalise the outlet flow rates to place the stagnation point at the centre. Therefore, the possibility of trapping objects in a microfluidic trap with two constraints (fixed and variable) is higher than that with only one variable constraint.

In reality the membrane is only able to partially block the channel to a certain height. The grey region in figure 5.9 shows the region that cannot be reached in experiments.
Figure 5.9: The effect of the normalised height \( h^* \) (the right outlet in figure 5.8) on the relative flow rate. The solid line shows the relative flow rate variations when there is a fixed constraint on the other outlet (the outlet in figure 5.8). The dash line shows the variation with the absence of the fixed constriction on the other outlet. In accordance with Tanyeri et al. (2011a), the grey region is not achievable as the membrane valve cannot block the cross-section more than a certain amount.

5.2.5 The effect of the relative flow rate on the position of the stagnation point

To find the relation between the outlet flow rates and the position of the stagnation point, an arbitrary position of the stagnation point \( Y_o \), shown in figure 5.10a, is considered. Then, by integrating the velocity field corresponding to each outlet, the flow rate of each outlet is yielded as the function of the stagnation point:

\[
Q_v = \int_{-w/2}^{Y_o} dy \int_0^h u(y,z)dz, \quad Q_c = \int_{Y_o}^{w/2} dy \int_0^h u(y,z)dz. \tag{5.15}
\]

Therefore, the ratio of the outlet flow rates as a function of the stagnation point position is obtained by (Tanyeri et al., 2010):
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\[
\frac{Q_v}{Q}(Y_o^*) = Y_o^* + \frac{1}{2} \left\{ 1 - \sum_{n,\text{odd}}^{\infty} \frac{192\alpha}{(n\pi)^5} \left[ \frac{\sinh \left( \frac{n\pi Y_o^*}{\alpha} \right)}{\cosh \left( \frac{n\pi}{2\alpha} \right)} + \tanh \left( \frac{n\pi}{2\alpha} \right) \right] \right\} \bigg(5.16\bigg)
\]

where \( Y_o^* = Y_o/w \) is the normalised stagnation point position along \( y \)-direction (extensional axis). Figure 5.10b shows the linear behaviour of the stagnation point position variations at different relative flow rates. As previously mentioned, in experiments the membrane is only able to partially block the channel to a certain height. The experimentally obtainable variation of the normalised channel height \( h_v^* \) is replotted in figure 5.10b to show the region where the stagnation point can be displaced (the region between the two red dash lines). Computing \( h_v^* \) as a function of \( Y_o^* \) (the blue line) was carried out by obtaining the \( Q_v/Q \) as a function of \( h_v^* \) from figure 5.9 and substituting them in equation 5.16.

### 5.2.6 Estimation of the required pressure to reposition the stagnation point

To estimate the required gas pressure in the control channel to reposition the stagnation point, the relation between the pressure and the height of the variable constraint is required. In this work, we adopted the valve response data (shown in figure 5.3c) by Tanyeri et al. (2010), i.e. the pressure in the control channel at different percentage of the valve opening \( (h_v^*) \). For the membrane thickness of 66 \( \mu \text{m} \) and the ratio of the PDMS base to cross-linker of 1:15 the data is shown in figure 5.11a. A linear response can be seen where the valve opening is more than 30%.
(a) An arbitrary stagnation point position in the cross-junction, where the inlet flow \((Q/2)\) bifurcates into the two outlets, \(Q_c\) and \(Q_v\). The distance between the centre line of the channel and the stagnation point along the \(y\)-direction is \(Y_o\).

(b) The black line (with the corresponding black left and bottom axes) shows the variation of the stagnation point at various relative flow rates. The blue line (with the corresponding blue right and top axes) indicates the region of height variation that is experimentally achievable (previously shown in the white region in figure 5.9). Computing \(h_v^*\) as a function of \(Y_o^*\) (the blue line) was carried out by obtaining the \(Q_v/Q\) from figure 5.9 and substituting them in equation 5.16, thereby indicating the region (between the two red lines) where the stagnation point can be experimentally displaced.

**Figure 5.10:** The effect of the relative flow rate \((Q_v/Q)\) and the normalised height of the variable constriction \((h_v^*)\) on the normalised stagnation point position \((Y_o^*)\).

Eventually, using the data provided in figure 5.10 \((h_v^*\) as a function of \(Y_o^*)\) and figure 5.11a \((P\) as a function of \(h_v^*)\), the variations of the stagnation point position at different control channel pressures \((Y_o^*\) as a function of \(P\)) were obtained and are shown in figure 5.11b. This graph is used as the calibration curve of the microfluidic trap.

It should be noted that in the experiments conducted in this thesis, this curve was obtained experimentally (to be described in section 5.4).
(a) The percentage of the valve opening at different gas pressures in the control layer for a PDMS membrane with a thickness of 66 µm and a ratio of the base to cross-linker of 1:15 (Tanyeri et al., 2010).

(b) Calibration Curve: The position of the stagnation point at different control channel pressures. The green line shows the linear trend line fitted to the linear part of the curve. The region between the two green dash lines is the region that the valve responds linearly.

5.2.7 Prediction of the trajectory of a target object in the microfluidic trap

Using the estimated calibration curve (figure 5.11b) the trajectory of the target that enters the cross-junction from an arbitrary location can be predicted. Based on the centroid position of the target and using the equation 5.2, the gas pressure in the control layer is updated, thereby displacing the stagnation point to a new location.

As previously mentioned, for using equation 5.2 the conversion factor \( K_C \) is required. This factor \( K_C \) is the slope of the fitted linear line in figure 5.11b. The equation of the fitted line \( Y_o^* = -0.0459P_{val} + 0.48238 \) is used to determine the position of the stagnation point. In other words, the stagnation point position is determined using (a linear interpolation of) the calibration curve.

In this simulation, the stagnation point is repositioned at \( f_{sys} = 30 \) Hz, which is the frequency of the control system. In an experiment, control system delay
time $\Delta t_{\text{delay}} = f_{\text{sys}}^{-1}$ is the period of time during which inline image processing is performed to detect the target plus the period of time the pressure valve operates to regulate the pressure. The lower the delay time, the greater the possibility of trapping objects at higher strain rates.

Figure 5.12 shows the trajectory of the targets in the cross-region during which the trapping was simulated. Normalised $x$ and $y$ axes are shown as $x^* = x/w$, $y^* = y/w$ in this figure. As can be seen, when the strain rate or $y_{t0}$ (the initial distance along the extensional axis between the target and the centre line of the channel) increase, the trap is not able to confine the target, e.g. ($\gamma = 20 \, s^{-1}$, $y_{t0} = -10 \, \mu m$) and ($\gamma = 15 \, s^{-1}$, $y_{t0} = 25 \, \mu m$). However, if in these two cases either the strain rate or the $y_{t0}$ decrease, it is possible to trap the targets i.e. ($\gamma = 15 \, s^{-1}$, $y_{t0} = 10 \, \mu m$) and ($\gamma = 2.5 \, s^{-1}$, $y_{t0} = 25 \, \mu m$). In these two cases, although the targets were experienced some fluctuations (shown in figure 5.12b), both cases confirm the convergence of the target at the trap centre.

(a) Trajectory of the target objects entered the cross-region at different initial locations and the strain rates of the flow.

(b) Variations of the locations of the target objects along the extensional axis entered the cross-region at different initial locations and the strain rates of the flow.

Figure 5.12: Predicted trajectory of five targets that entered the cross-region at different locations and strain rates during trapping.
In figure 5.13, the required pressure and pressure differences in the control channel are shown. The pressure changes for these targets in figure 5.12 were between about 4 to 13 psi and the maximum pressure difference required to trap objects was about 3 psi. This information assists in selecting the electronic pressure valve for the experiments.

Considering the data in this simulation, it can be confirmed that trapping objects entering at \((-20 < y_0 < 20)\) and the strain rate below 10 s\(^{-1}\) is possible. Although we intend to expose high strain rates to the microorganisms, it is not initially necessary to deliver the fluid at a high flow/strain rate. In experiments, the pressure regulator responds faster for smaller pressure changes, and this is typically more relevant after the initial confinement of a target (shown in figure 5.13). Therefore, it is possible to confine a target at a low flow rate and then gradually increase the flow rate/strain rate. In this case, smaller pressure changes are required to bring the target back to the trap centre when the target is displaced.

![Graphs showing pressure and pressure difference](image)

(a) The pressure in the control channel to trap the targets in figure 5.12.

(b) The pressure difference in the control channel to trap the targets in figure 5.12.

Figure 5.13: Required pressures and pressure differences for trapping the five targets in figure 5.12.
5.3 Active flow control experimental setup and procedure

5.3.1 Imaging setup

The Nikon microscope used in the micro-PIV experiments (described in section 4.2) was upgraded to a double-turret microscope, in which simultaneous imaging with two cameras from the side port and the back port of the microscope was possible. This configuration enabled us to stream the images using a low resolution and relatively a high frequency camera to control the flow, and simultaneously acquire high definition movies using a DSLR camera. A low-resolution monochrome PointGery camera (Blackfly 0.3 MP, GigE PoE) capable of streaming images at 90 fps at full resolution was coupled to the C-mount back port of the microscope using a CS-mount to C-mount adaptor and a 0.5× relay lens. The PIV camera (in section 4.2) was replaced by a Nikon D800 camera to acquire high resolution colour images at 50 fps. Similar to the micro-PIV experiments (in section 4.2) due to the large camera sensor (35.9 × 24.0 mm²) vignetting was inevitable. However, in contrast with the micro-PIV experiments, we used a 2× universal DSLR adapter (Spot Imaging). The aberration that the adaptor produces is acceptable for the purpose of video creation, but not for micro-PIV measurements. Combinations of hollow tubes and 2× teleconverters were used where higher magnifications were required. It is not possible to increase the magnification by replacing the microscope objective, as FOV of the control camera is decreased and it is not possible to acquire the image of the whole cross-region to detect the targets.

The microscope was equipped with the same 20× objective lens (CFI S Plan Fluor ELWD) that was used in the micro-PIV experiments (in section 4.2). All G-2A in micro-PIV setup (section 4.2.1) was replaced by a filter cube including only a 50/50, 45° mirror to split the light between the two cameras. No extra light source
was used to illuminate the flow for imaging apart from the ambient room lights. Figure 5.3 shows the schematic of the control system setup.
Figure 5.14: Schematic of the control experimental setup.
5.3.2 Online control setup

The microfluidic trap is controlled by a custom-built MATLAB code (developed by the author) in which inline image processing and controlling of the devices are carried out.

The control (PintGrey) camera is directly controlled in the MATLAB environment, which takes an image when required and streams it to a computer using a PoE (Power over Ethernet) cable.

To control the electronic pressure regulator and the syringe pump, a simple Arduino UNO controller board was employed. The controller is used to send 0-5 volt digital signals from MATLAB through its pulse-width modulation (PWM) pins. The signal is amplified to a 0-10 volt signal, suitable for the input of the pressure regulator.

To pressurise the control channel, a 2000 psi Nitrogen tank was used to supply the pressurised Nitrogen. Using a manual valve (Gascom) the pressure at the outlet of the tank is reduced to 33 Psi, suitable for the inlet of the electronic pressure regulator. A high resolution electro-pneumatic pressure regulator (Proportion-Air, QPV series) with the accuracy and resolution of \( \pm 0.2\% \) and \( \pm 0.005\% \), respectively, at full scale was calibrated to provide the outlet pressure at 0-30 psi. This covers the range of the required pressure in the simulation (previously shown in figure 5.13). The pressure regulator is capable of responding for a pressure change of 1 psi, within 5 and 11 ms when pressurising and relieving the pressure, respectively. This leads to an upper limit of roughly 90 Hz (the same as camera frequency) for the response time, given that one only needs to change the pressure by 1 psi to keep a particle at the stagnation point. However, according to the simulation, after the initial confinement of a target, less than 0.5 psi is sufficient to trap the targets. This assists us in estimating the time delay of the control system (to be described in section 5.3.3)
As PDMS is a gas permeable material, if the control channel is pressurised directly with Nitrogen, the gas permeates to the fluidic channel, which results in disrupting the water flow and control system. To tackle this problem, prior to establishing the connection between the pressure valve and the control channel, it was filled with Lavandula essential oil (i.e a type of vegetable oil). The same PFA tubes (0.020 inch ID, 1/16 inch OD) and the same 24 gauge bent metal tubing (explained in section 3.2.4) were used to establish the connection between the electronic pressure regulator and the control layer.

5.3.3 Inline image processing

In the image processing field, the foreground detection is carried out by comparing the pixels of an image to a previously defined background image, followed by deciding whether the pixels of the image belong to the background or the foreground (Bouwmans et al., 2014).

The accuracy of foreground detection depends on how much the foreground is distinguishable from the background. In most cases, additional image processing steps are required to achieve the best possible foreground detection results (Bouwmans et al., 2014). The image processing methods in foreground detection are usually tailored to the light condition, the morphology of objects and the consistency of the background.

In this work, the foreground detection was carried out using the “Computer Vision System” toolbox in MATLAB. This includes acquiring the cross-region as the background and comparing it with the image of the target in the cross-region. Image comparison was followed by determining whether each pixel belongs to the background (channel) or the foreground (target). Before and after the foreground detection, image contrast enhancement and some morphological operations were carried out to improve locating the accuracy target’s centroid.
There are two important factors that should be considered in such experiments. Firstly, as a target will eventually stay in the trap centre and become a stationary object, no adaptive backgrounding should be carried out, otherwise the trapped object will eventually become part of the background and it will no longer be possible to locate its centroid. Secondly, as we acquire images of cyanobacteria filaments with very small cell connections using a low resolution camera, we should ensure that the connections of the filaments are labelled as the foreground, otherwise a dramatic discrepancy between the computed centroid and real centroid of the filament will occur.

Figure 5.15: Implementation of the image processing steps that were used for foreground detection (detecting the *A. circinalis* filament in the microchannel).

Figure 5.15 depicts the implementation of the image processing steps used in the present work to locate the centroid of the *A. circinalis* filament. Firstly, the background image of the region of interest (ROI) was acquired. This was conducted
prior to the filament entering ROI (partially shown in figure 5.15a). Figure 5.15b shows a filament of the cyanobacteria in the ROI. Upon acquiring the image of the target (filament) the image contrast was enhanced to increase pixel intensity of the filament (shown in figure 5.15c). Afterwards, the foreground was detected. Foreground detection requires defining a threshold parameter that depends on the light condition to determine whether each pixel belongs to the channel or the target. The background was then subtracted from the foreground, thereby achieving the target object mask (the binary image shown in figure 5.15d). This step was followed by removing small objects and noises, by defining a certain threshold and removing the blobs (connected white regions) smaller than the threshold (shown in figure 5.15e). As discussed in the preceding paragraph, due to the low resolution of camera and the shape of the objects, detecting the connections of the filaments is challenging. As can be seen the connections are neither observable in figure 5.15b, nor detectable in figure 5.15e. To alleviate this issue, we used morphologically close operation by a $7 \times 7$ pixel rectangular-shaped structural element to increase the connectivity of the blobs without over-fattening it. Stated in Smith et al. (1997), morphologically close operation in processing of a binary image includes a morphologically erosive operation (making the blob smaller) followed by a morphologically dilative operation (making the blob larger). Figure 5.15f shows the post-processed mask of the filament after applying the morphologically close operation. Eventually, the centroid of the filament was determined using the blob analysis of the MATLAB computer vision system toolbox.

In the present experiments, the time period of the image processing operations was about 20 ms, which results in the maximum system delay time $\Delta t_{delay} \approx 30$ ms, considering the response time of the electronic pressure regulator.
5.4 Calibrating the microfluidic trap

As mentioned in section 5.2.6, the calibration curve of the microfluidic trap is the variation of the stagnation point position at different control channel pressures. The linear region of this curve is used to determine the conversion factor ($K_C$ in equation 5.2). To experimentally determine this curve, a fluid visualisation experiment was conducted, which delivered the fluid seeded with tracer particles (used in section 4.3) to the cross-junction. By capturing the images of the cross-region, the flow is visualised. In this experiment, the flow was illuminated using the Nikon microscope Epi-fl illuminator (Mercury lamp). The epifluorescence imaging setup configuration that was used in micro-PIV experiments (in section 4.2.1), except the PIV camera that was replaced by the control camera was employed to acquire the images. The backgrounds (out-of-focus particles) of the acquired images (using the method explained in 4.7.1) were then removed and then 150 images were overlaid. This procedure was repeated at the different pressures in the control channel, and for each set of images the stagnation point was determined manually/visually with the accuracy of one pixel. One pixel corresponds to one micron for the control camera used in this experiment.

Figure 5.16 shows an example of 150 overlaid images at the flow rate of 50 µL/hr and control channel pressure of about 8 psi. The distance of the stagnation point position from the centre line of the channel along the extensional and compressional axes are represented by $Y_o$ and $X_o$, respectively. As can be seen the stagnation point is not located on the vertical centre line, due to the flow added to one of the inlets where the sample injection port is located (shown in figure 3.2). However, $X_o \neq 0$ only occurs when the injection port is open. In trapping experiments once a target is trapped, the injection port is manually closed and the stagnation point gradually moves on the centre line along the compressional axis. Figure 5.17 shows the calibration curve that was experimentally determined (at 50 µL/hr)
Figure 5.16: An example image of 150 overlaid recordings (at the flow rate of 50µL/hr and control channel pressure of about 8 psi) that was used to determine the calibration curve of the microfluidic trap.

at different pressure in the control channel. The slope of the fitted trend line represents the conversion factor ($K_C$).

\[ Y_o = -101.4091 P + 806.3129 \]

Figure 5.17: Experimentally determined calibration curve of the microfluidic trap. The green dash line is the trend line fitted to the linear region, and the slope of this line represents the conversion factor ($K_C$).
5.5 Active flow control experiment

The procedures described in sections 4.5 and 3.2.4 were implemented to mount the microfluidic device onto the microscope stage and to establish the connection between the microchannel and syringes, respectively. The 1000 \( \mu \text{L} \) syringe (connected to the buffer inlets) and the 250 \( \mu \text{L} \) syringe (connected to the injection port) were filled with water and a cultures medium (e.g. MLA medium containing \( A. \text{circinalis} \)), respectively. The control channel was filled with essential oil. Using a 1 mL plastic syringe with a 24 gauge blunt syringe needle the oil was injected into the control channel through one of its ports (e.g. the right-hand side port schematically shown in figure 5.3c). When the channel was filled and the oil overflowed from the other port, the plug was placed to block the channel (the left port schematically shown in figure 5.3c). The syringe and its needle were then replaced by the connections to the pressure regulator.

Prior to operating the control system, the same procedure in micro-PIV experiments (previously described in 4.5) was used to remove all the bubbles from the fluidic channel. Prior to injecting the targets, the control system was operated to compute the background (cross-region) and then the shut-off valve was manually
opened to inject the targets. Once a target was trapped, the valve was closed manually. After about one minute of trapping, the flow rate was either kept unchanged or was increased using the functions shown in figure 5.18. The right-hand side axis of the figure shows the strain rate of each flow rate obtained from figure 4.16/equation 4.15 in micro-PIV experiments described in the preceding chapter. The functions were defined within the pump inbuilt-interface. Three different functions (cases) were defined: In Case 1, the flow rate remains unchanged (at the flow rate and strain rate of $Q = 50 \mu$L/hr and $\gamma = 2.3 \text{ s}^{-1}$, respectively). Case 2 is defined as a step function that the flow rate increases by $Q = 25 \mu$L/hr every five seconds. The same step function is defined in Case 3, however, the flow rate is kept constant at ($Q = 400 \mu$L/hr and $\gamma \approx 20 \text{ s}^{-1}$). The pump was connected to the Arduino controller, thereby sending signals to MATLAB and recording the exact time of triggering the flow rate functions.

5.6 Growth conditions for the algae

Although we focus on *A. circinalis*, we grew, trapped and exposed other fresh water species (*Pseudanabaena limnetica* and *Pseudanabaena galeata*) of cyanobacteria/blue-green algae to the straining flow, to compare the results with *A. circinalis*. We also trapped and exposed *Dunaliella tertiolecta* (the single-celled motile marine species) only to demonstrate the capability of the microfluidic trap to confine both motile and non-motile microorganisms.

All freshwater species were grown in an MLA medium (provided by CSIRO), while the single marine species was grown in a modified “f-medium” (Guillard and Ryther, 1962). Individual cultures were maintained in 25 mL Tissue Culture Flasks (TCFs) – canted neck, non-pyrogenic, sterile, polystyrene flasks (Corning Inc.). The cultures were grown at room temperature ($22^\circ C \pm 2^\circ C$), under cool white fluorescent lights with a 13:11 light dark cycle and photon flux density of $\sim 40 \mu$Einsteins.
Two strains of *Anabaena circinalis Rabenhorst* strains with CSIRO IDs of ACMR01 and ACCR03 were acquired from the CSIRO (Commonwealth Scientific and Industrial Research Organisation) in September 2014. The former, shown in figure 5.19a, was isolated (by CSIRO) from Canning River, Perth, Western Australia, whereas the latter, shown in figure 5.19b, was isolated from Murrumbidgee River, New South Wales, Australia. The *Pseudanabaena limnetica* and *Pseudanabaena galeata* were isolated (by Dr. David Hill, University of Melbourne) from wash water at the Tarago Water Treatment Plant in Gippsland, Victoria, Australia in
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5.7 Results and discussions

Here we characterise the trajectory of the target objects in the microfluidic trap and present the observations of the straining flow effect on the cyanobacteria.

5.7.1 The experimentally determined trajectory of the trapped target objects

Figure 5.22 shows three partially overlaid images of the *A. circinalis* strain (ACMR01) that entered the cross-slot region and eventually was trapped in the cross-slot centre. The trajectory of the filament’s centre of mass is superimposed on the images and compared with the estimated trajectory of an object in the straining flow without active control (obtained from micro-PIV results in section 4.11.1).
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As previously mentioned, with the intention of demonstrating the capability of the microfluidic trap for confinement of both motile and non-motile target objects, we also trapped *D. tertiolecta* species. Figure 5.23 shows a trapped *D. tertiolecta* cell and its trajectory in the microfluidic device. The experimentally determined trajectory of the *D. tertiolecta* agreed well with the simulation results (shown in
Figure 5.23: Eleven partially overlaid images of the confinement of a *Dunaliella tertiolecta* in the cross-slot junction using an active control system at $Q = 100 \mu\text{mL/hr}$. The experimentally measured target’s trajectory is shown by white circles, whereas the black dash line shown the numerically estimated target trajectory. The red dash line shows the streamline of the flow with the absence of the control system.

The discrepancy might be rooted in the fact that in the simulation, the target object is simulated by its centroid (a point moving in the straining flow). However, the relatively large size of the *A. circinalis* and its filamentous morphology results in disrupting the hyperbolic streamlines of the flow, whereas the *Dunaliella*, which is much smaller than *A. circinalis* and spherical in shape, follows the flow streamlines with less influence on them. Therefore, there was less discrepancy between the simulation and the experimental results. In the next chapter, using simultaneous micro-PIV measurements and an active flow control experiment in the cross-slot microchannel, the flow field around a filament of *A. circinalis* in the cross-region will be measured. This will enable us to characterise the flow around...
the target filaments while it is trapped.

5.7.1.1 Fluctuations of the trapped target along the extensional axis

In principle, the position of a trapped sub-micron object fluctuates due to the Brownian motion. In the present experiments, due to the relatively large size of the trapped objects, the Brownian motion does not influence the trapped targets. However, fluctuations in the centre of mass position of a target object can be seen in figure 5.24a. The figure shows the centre of mass position of the *A. circinalis* filament along the extensional axis ($Y_{tp}$) over the last 2.5 minutes of its confinement. The fluctuation signal is also rescaled over six seconds and shown in the inset plot of figure 5.24a. These fluctuations occurred due to the slightly inaccurate detection of the target’s centroid.

As stated in section 5.3.3, the centre of mass of the target object is determined using image processing techniques, and the accuracy of determining the centre of mass of the object is dependent on how much the foreground is distinguishable from the background. Due to the real-time control experiments and the necessity of having relatively high speed operations, in such an experiment it is not efficient to maximise the number of time consuming image processing techniques to enhance foreground detection quality. Reducing the image processing techniques caused slightly inaccurate determination of the centre of mass of the target, but sufficiently accurate for the purpose of confinement. The inaccuracy resulted in the continuous small fluctuation of the target object (figure 5.24a).
(a) Centre of mass position of the filament ($Y_{tp}$) along the extensional axis over the last 2.5 minutes of its confinement and the rescaled ($Y_{tp}$) over six seconds in the inset plot.

(b) Energy spectrum of the centre of mass positions of the filament ($Y_{tp}$) and the fitted Lorentzian function and corner frequency.

**Figure 5.24:** Characterisation of the centre of mass position of a trapped $A.\ circinalis$ filament along the extensional axis ($Y_{tp}$).
The histogram and standard deviation of the signal are shown in the inset plot of figure 5.24b. Similar to the work carried out by Tanyeri et al. (2010), the histogram of the signal is close to a fitted normal distribution. However, the standard deviation was $\sigma_{Y_{tp}} = 1.91 \, \mu m$ that is three times the value reported by Tanyeri et al. (2010) for a two micron trapped fluorescent bead at 2.5 times lower strain and flow rates. It should be noted that the fluctuations of the bead occurred due to the effect of the Brownian motion, as locating a fluorescent by fitting a point spread function to the particle emission intensity can accurately determine the position of the bead (Tanyeri et al., 2010), whereas, the fluctuations of $Y_{tp}$ in the present work stems from the image processing method. Figure 5.24b shows the single-sided energy spectrum of the same $Y_{tp}$ signal shown in figure 5.24a.

### 5.7.1.2 Calculation of the trap stiffness

As mentioned above, Brownian motion leads to continuous vibration of a sub-micron trapped bead. Brownian motion is essentially white with constant amplitudes. The equation of the motion of a trapped sub-micron object (by ignoring the inertial forces due to the low Reynolds number) is given by (Shaevitz, 2006):

$$\zeta \ddot{x}(t) + \kappa_t x(t) = F_n(t), \tag{5.17}$$

where $x(t)$ is the position of object, $\zeta$ is hydrodynamic drag coefficient, $\kappa_t$ is the trap stiffness and $F_n(t)$ is the noise source (Brownian for the sub-micron object). Using Fourier transform of the equation the energy spectrum is given by (Shaevitz, 2006):

$$|\tilde{x}(f)|^2 = \frac{|\tilde{F}_n(f)|^2}{4\pi^2 \zeta^2 \left[ \left( \frac{\kappa_t}{2\pi \zeta} \right)^2 + f^2 \right]}, \tag{5.18}$$
where $|\tilde{x}(f)|^2$ is the magnitude of Fourier transform of the position of the object, and $\kappa_t$ is the trap stiffness. The Fourier transform of the Brownian motion $|\tilde{F}_n(f)|^2$ is constant (white noise) and equal to $4\zeta K_B T$.

Therefore, the energy spectrum of the positions of the trapped sub-micron target is a $f^{-2}$ noise (brown noise) and corner frequency is equal to \( \left( \frac{\kappa_t}{2\pi\zeta} \right) \) and therefore trap stiffness is given by (Shaevitz, 2006):

$$\kappa_t = 2\pi\zeta f_c$$  \hspace{1cm} (5.19)

As discussed above, in our case the source of noise is not the Brownian motion and it was caused by the inaccuracy of the target detection. However, the same behaviour (brown noise) is anticipated as the target position randomly vibrated with zero mean. To prove this, we fitted different curves including $f^{-2}$ noise (brown noise), $f^{-1}$ noise (pink noise). As our target object may not be considered massless (similar to sub-micron beads) and despite using the low Reynolds number, we also considered inertial forces in the motion of equation that results in a curve consisting $f^{-4}$ and $f^{-2}$. However, amongst all the curve fitted (not shown) to the energy spectrum we found that the $f^{-2}$ noise (figure 5.24b) agrees more with the energy spectrum. Therefore, the trap stiffness is calculated using the same equation as equation 5.19. The corner frequency of the signal (here $f_c = 0.12$ Hz) was then determined by fitting a Lorentzian function with an Levenberg-Marquardt algorithm in the least square sense to the energy spectrum (figure 5.24b).

To calculate the trap stiffness using equation 5.19 the hydrodynamic drag coefficient is required (drag force divided by velocity). For a spherical particle immersed in a uniform Stokes flow, it is given by $\zeta = 3\pi\mu d_p$. However, in case of trapping objects in the straining flows and in particular for the non-spherical objects or
slender bodies $\zeta = 3\pi \mu d_p$ cannot be used. In the next chapter, using a combination of the experimental results and analytical solution of the straining flow over an ellipsoidal object we calculated the exerting force to *A. circinalis* filaments (approximated by ellipsoidal shape objects). Therefore, the hydrodynamic drag coefficient is calculated using the method to be described in section 6.5.2. Finally, the trap stiffness (using 5.19) is found to be $\kappa_t = 0.3$ pN/nm. It is comparable with, but lower than, the reported trap stiffness $\kappa_t = 1.2$ pN/nm by Tanyeri et al. (2010). The discrepancy is firstly rooted in the difference between the shape and the size of the trapped objects, and secondly the fact that they used the Stokes law to calculate the hydrodynamic drag coefficient. Whereas, we more accurately computed the hydrodynamic drag coefficient using our experimental measurement in the next chapter.

### 5.7.2 Observation of the algae in the controlled straining flow

![Image](image_url)

**Figure 5.25:** An example of a filament of *A. circinalis* (ACCR03) exposed to the straining flow at $Q = 50$ $\mu$L/hr and $\gamma = 2.3$ s$^{-1}$ for about an hour. No mechanical damage to the filament was observed.

With the intention of observing mechanical damage to filaments/cells of *A. circinalis* by the straining flow, we trapped a large number of filaments of *A. circinalis*
in the microfluidic trap and exposed them to the straining flow with different stain rates (shown in figure 5.18). Figure 5.25 shows an example of a filament of *A. circinalis* after approximately one hour of confinement in the straining flow (case 1 in figure 5.18, $Q = 50 \mu$L/hr and $\gamma = 2.3 \text{ s}^{-1}$). As can be seen no breakage of, or mechanical damage to, the strain was observed and this was the case for all but one of the *A. circinalis* filaments.

As mentioned above, only one (out of hundreds) of the filaments of *A. circinalis* showed any changes to the cell-cell, connections when experiencing the straining flow with the strain rate of $\gamma = 2.3 \text{ s}^{-1}$ (shown in figure 5.26). Considering the filament in figure 5.26, it can be seen that the cell connection that is surrounded by a red circle appeared to be loosened, however, it remained unchanged after about three minutes of experiencing strain flow onwards.
Figure 5.26: The only filament of the *A. circinalis* (ACMR01) that showed some changes in the cells connections during the straining flow ($\gamma = 2.3 \text{ s}^{-1}$), however, it remained unchanged after about three minutes of experiencing strain flow.
Figure 5.27: An example of a filament of *A. circinalis* (ACMR01) in the straining flow exposed to the straining flow at $Q = 50 - 800 \mu$L/hr and $\gamma = 2.3 - 44 s^{-1}$. Although the straining flow affected the topology of the filament, no mechanical damage to the filament was observed.

The same results (with no mechanical damage) were observed when the filaments of *A. circinalis* were exposed to the increasing straining flow (Case 3: strain rate up to 20 s$^{-1}$ and Case 2: strain rate up to 42 s$^{-1}$ in figure 5.18). In the case of non straight filaments, the topology of the filaments slightly changed, and they became straighter. When the flow rate and strain rate decreased, they returned to their initial form. However, no change in the cells’ connections were observed. As an
example, figure 5.27 shows a filament of *A. circinalis* that experienced increasing straining flow (from 2.3 to 41.9 s\(^{-1}\)). In this experiment, we used the step function in Case 2 of figure 5.18, but the step function started after 10 minutes of trapping (rather than one minute as shown in figure 5.18).

Regarding the *P. limnetica* and *P. galeata*, although *Pseudanabaena* filaments look weaker than *Anabaena* with smaller cell connections and thickness, no mechanical damage due to the straining flow was observed (images are not shown).

### 5.7.3 Cultures grown in a low-nitrate MLA medium

We also grew the *A. circinalis* in a low-nitrate medium (initially 10% of the original nitrate concentration in the MLA medium. This resulted in the poor health conditions of the filaments and the existence of necrotic (dead) cell cells in the filaments. In contrast with the observed results in the previous section, the cultures that were grown in the low nitrate medium appeared to be broken when they entered the cross-region (at the lowest strain rate 2.3 s\(^{-1}\)) and before trapping in the trap centre.

In these experiments we have not carried out viability tests or chlorophyll measurements. However, the colour of the filaments depicts the health condition of the cultures to some extent. Change in their colour from green to yellow or orange is a reaction to the lack of nitrate in the medium and indicates some suffering.

Contrary to the previous section the filaments in this experiment were mainly orange and in some cases with distinct necrotic cells (e.g. figure 5.28b). Figure 5.28b shows a filament of *A. circinalis* entered the cross-region. One of the junctions of the necrotic cell in the filament appeared to be broken before entering the region. This trend can be seen in many other examples of the cultures grown in this low-nitrate medium. Figure 5.29 and 5.30 show more examples of suspected mechanical damage to the filaments grown in the low-nitrate medium.
Figure 5.28: A filament of *A. circinalis* grown in the low-nitrate medium with poor health condition that have a distinct necrotic (dead) cell appeared to be loosened or broken from one of the necrotic cell junction when entered the cross-junction. The filament finally was divided to two separate filaments.
Figure 5.29: An example of a filament of *A. circinalis* grown in the low-nitrate medium with poor health condition appeared to be loosened or broken from one of its junctions when entered the cross-region. The filament finally was divided to two separate filaments. The image included four partially overlaid images of the filaments at four different location of the cross-junction (*Q* = 50 µL/hr).

Figure 5.30: An example of a filament of *A. circinalis* grown in the low-nitrate medium with poor health condition appeared to be loosened or broken from one of its junctions when entered the cross-region. The filament finally was divided to two separate filaments. The image included four partially overlaid images of the filaments at four different location of the cross-junction (*Q* = 100 µL/hr).
5.8 Summary

The cross-slot channel was equipped with an image-based real-time feedback control system to expose cyanobacteria to quantified straining flows to investigate the possibility of mechanical damage to cyanobacteria. The implementation and design of the microfluidic trap were detailed. Prior to the design of the microfluidic trap, the control system and its effect on the flow were simulated, thereby predicting the trajectory of a target object in the microfluidic trap. Later the results of the experiments were compared with those of the simulation, showing good agreement. The fluctuations at the centre of mass position of the trapped objects were characterised and the trap stiffness was determined.

Regarding the effect of straining flow on the algae, no mechanical damage was observed for healthy cyanobacterial filaments, while, those filaments that were grown in the low nitrate medium, and particularly the ones that had necrotic (dead) cells in their filaments, appeared to be broken when exposing to straining flows at $\gamma = 2.3 \, s^{-1}$. 
Chapter 6

Simultaneous micro-PIV measurements and active control in the cross-slot channels

In this chapter we report a novel experiment in the cross-slot microfluidic trap in which micro-PIV measurements and real-time control are simultaneously carried out in order to measure the velocity field around a trapped target. This experiment enables us to compute the flow stresses and the exerting forces on a trapped target and also visualise the flow around it.

Calculation of the exerting force and measuring the deformation of a target object (using imaging) enables one in principle to compute the stiffness of the target. In the preceding chapter we observe neither any mechanical damage to, nor any dramatic deformation in the filaments of cyanobacteria. Therefore, at present it is challenging to obtain the mechanical properties of the cyanobacteria from the experiments. However, the present experiment opens new vistas for future experiments in the straining microfluidic trap when calculation of the flow stresses and the resulting forces on a target object is required. This can be exemplified by trapping easily deformable objects such as bubbles (Ulloa et al., 2014), DNA
molecules (Li et al., 2015) or blood globules (Kim et al., 2015), when they are exposed to the controlled straining flow. In this work we approximated a filament of cyanobacteria with a prolate spheroid (an elongated ellipsoid of revolution). However, the present method can be used to compute the exerting force on any trapped object that can be approximated with a tri-axial ellipsoid.

In order to calculate the exerting force on an ellipsoidal-shaped object in the straining flow, a high-resolution three-dimensional velocity field is required to accurately compute the velocity gradients. In the microfluidic trap, the necessity of having a relatively large field of view in the control camera (i.e. the whole cross-junction) limits the use of high magnification microscope objective lenses. Therefore, it is not possible to simultaneously control the flow in the cross-slot channel and measure the velocity field with high resolution near the trapped object’s body. Moreover, classical micro-PIV measurement techniques are limited to measuring a two-dimensional velocity field. Hence, apart from the PIV results, we adopt the analytical solution of the flow around an ellipsoidal-shaped object in the straining flow to calculate the velocity gradients. However, it should be noted that one cannot solely rely on the analytical solution of the straining flow over an object, as analytical solution requires the strain rate of the undisturbed flow (away form the trapped object). Although in the previous chapter we assumed that when the objects are trapped, the strain rate in the flow is the same as the strain rate of the flow with the absence of the target objects, this assumption is suitable only when the size of the target object is very small compared to the cross junction (e.g. trapping the Dunaliella tertiolecta in the previous chapter). In the case of trapping of relatively large objects such as Anabaena circinalis the average flow strain rate (obtained from the experiments to be explained later in this chapter) is higher than flow strain rate with the absence of the target, due to obstruction from the target object. Hence, using the new micro-PIV measurements we obtain the strain rate with the presence of the target object and substitute it in the analytical solution of the flow over an ellipsoidal object immersed in a creeping
straining flow, presented by Jeffery (1922). Upon ensuring that the analytical and experimentally measured velocity around the ellipsoid agree, we use the stresses on the surface of the ellipsoid obtained from the analytical solution to calculate the force exerted by the flow on the object. In other words, by combining the micro-PIV results and the analytical solution, we obtain the flow stress tensor over a trapped object.

### 6.1 Basic concepts of simultaneous micro-PIV measurement and real-time control

As it is noted in section 4.1, the use of volume illumination in micro-PIV measurements results in bright background in the images that drastically reduce the signal-to-noise ratio and the correlation peak detectability. Therefore, the epifluorescence imaging technique is implemented to alleviate this issue. In the micro-PIV measurements conducted in chapter 4, a 532 nm double-pulsed laser light source was used to illuminate the tracer particles. In chapter 5, using an image-based active control system in the cross-slot channel, algae filaments were trapped at the stagnation point of the straining flow. In those experiments a constant white light source (ambient room lights) was used for imaging.

Simply combining the two experimental steups (micro-PIV setup shown in figure 4.2 and microfluidic trap setup shown in figure 5.14) to conduct simultaneously micro-PIV measurements and real-time control in the microfluidic trap leads to two major problems. The combination of the two setups is shown in figure 6.1. The first issue is the disruption of the control camera by the laser pulses. The second issue is the production of extremely bright background in the images acquired by the PIV camera sensor due to the constant white light source. The former results in preventing the detection of a target object in the cross-slot channel. The top inset image in figure 6.1 (figure 6.1a) shows an example of the image captured by
the control camera when the laser was pulsing. As can be seen, the laser pulse interferes with the image processing techniques for object detection. While, the latter is the production of extremely bright background that precludes correlating the recorded images. The bottom inset image in figure 6.1 (figure 6.1b) shows an image of the particles captured by the PIV camera, while the constant white light was illuminating the flow.
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Figure 6.1: Schematic of the combination of the micro-PIV and active control setups and the resulting imaging problems in the images acquired by the PIV and the control camera.
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Figure 6.2: Schematic of the modified simultaneous micro-PIV and control experimental setups.

(a) An image captured by the control camera
(b) A pair of overlaid images captured by the PIV camera
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To tackle the first issue we replaced the continuous white light with a continuous blue light source (i.e. filtering all the wavelengths except blue). Whilst, to address the second issue, we filtered all the wavelengths (except blue) hitting the control camera sensor. Using this arrangement, the control camera sensor does not capture the laser pulses, and the PIV camera does not acquire the bright background caused by the constant light source. Figure 6.2 shows the schematic of the combined micro-PIV and active control system setups, where the two problems that were pointed out are solved using two single-band (blue) bandpass filters. The bandpass filters block all the wavelengths out of the range of 420-480 nm. Figure 6.3 shows the diagram of the fluorescent intensity of the particles and the transmittance of the filters that is used to simultaneously conduct micro-PIV measurement and real-time control experiment in the microfluidic trap.

![Diagram showing excitation and emission of fluorescently labelled particles and transmittance of barrier and excitation, single-band bandpass filters and dichroic mirror.](image)

**Figure 6.3:** Excitation and emission of fluorescently labelled particles and transmittance of barrier and excitation, single-band bandpass filters and dichroic mirror (the data was received from Thermo Fisher and Nikon Instruments Co. and Semrock Inc.).
6.2 Simultaneous micro-PIV and active control experimental setup

All the components in the two experimental setups (micro-PIV measurements and real-time control experiments described in sections 4.2 and 5.3, respectively) were used except for a few minor changes. As can be seen in figure 6.2, a filter cube including the 45°, 50/50 mirror (that splits light to the control camera and PIV camera) and a single-band bandpass filter was placed into the upper turret of the microscope. While, the epifluorescent cube filter was placed into the bottom turret of the microscope.

As it is impossible to filter the ambient room lights, in order to filter the light wavelengths of the constant light source, instead of using the ambient room lights, a LumeCube light source (0-1500 Lumens) with a single-band bandpass filter attached in front was used to illuminate the flow. Figure 6.4 shows a photograph of simultaneous micro-PIV and real-time control experimental setup and its components used in this work.

6.3 Simultaneous micro-PIV and active control experiment

The 1000 µL syringe (connected to the buffer inlets) was filled with water and seeding particles, and the 250 µL syringe (connected to the injection port) was filled with the culture medium (the connection establishment were explained in section 3.2.4). The same procedure (explained in sections 5.5 and 4.5) was used to run the micro-fluidic trap and micro-PIV measurements. The pump was operated at the constant flow rate of 50 µL/hr.
Figure 6.4: Photograph of the simultaneous micro-PIV and real-time control experimental setup.
6.4 Analytical solution of the flow over an ellipsoidal object immersed in a creeping straining flow

Here we adopt the analytical solution of the flow over an ellipsoidal object immersed in a creeping flow, wherein each component of the velocity field is a linear function of the Cartesian coordinates, $x$, $y$ and $z$, presented by Jeffery (1922). We simplify the solution to the pure straining flow around an ellipsoid.

6.4.1 Confocal ellipsoidal coordinate

The confocal ellipsoidal coordinate is represented by Feshbach and Morse (1953):

$$\frac{x^2}{a^2 + \lambda} + \frac{y^2}{b^2 + \lambda} + \frac{z^2}{c^2 + \lambda} = 1,$$

(6.1)

where $a$, $b$ and $c$ are the length of the semi-principle axes of the ellipsoid along the $x$, $y$ and $z$ of the Cartesian coordinate, respectively. Also $\lambda$ is a constant that produces the surface of confocal ellipsoids in the ellipsoidal coordinate. The variables $\Delta$ and $\Upsilon$ used in the confocal ellipsoidal coordinate are defined as:

$$\Delta = \sqrt{(a^2 + \lambda) + (b^2 + \lambda) + (c^2 + \lambda)};$$

$$\Upsilon = \left(\frac{x^2}{a^2 + \lambda} + \frac{y^2}{b^2 + \lambda} + \frac{z^2}{c^2 + \lambda}\right)^{-\frac{1}{2}}.$$

(6.2)

The auxiliary variables in confocal ellipsoidal coordinates that are defined through the Jacobi elliptic integral transforms, are given by:
$\alpha = \int_\lambda^\infty \frac{d\lambda}{(a^2 + \lambda)\Delta}, \quad \alpha' = \int_\lambda^\infty \frac{d\lambda}{(b^2 + \lambda)(c^2 + \lambda)\Delta}, \quad \alpha'' = \int_\lambda^\infty \frac{\lambda d\lambda}{(b^2 + \lambda)(c^2 + \lambda)\Delta},$

$\beta = \int_\lambda^\infty \frac{d\lambda}{(b^2 + \lambda)\Delta}, \quad \beta' = \int_\lambda^\infty \frac{d\lambda}{(a^2 + \lambda)(c^2 + \lambda)\Delta}, \quad \beta'' = \int_\lambda^\infty \frac{\lambda d\lambda}{(a^2 + \lambda)(c^2 + \lambda)\Delta},$

$\Gamma = \int_\lambda^\infty \frac{d\lambda}{(c^2 + \lambda)\Delta}, \quad \Gamma' = \int_\lambda^\infty \frac{d\lambda}{(a^2 + \lambda)(b^2 + \lambda)\Delta}, \quad \Gamma'' = \int_\lambda^\infty \frac{\lambda d\lambda}{(a^2 + \lambda)(b^2 + \lambda)\Delta}.$

(6.3)

### 6.4.2 Velocity field around an ellipsoid immersed in the pure straining creeping flow

**Jeffery (1922)** solved the Stokes equations of the creeping flow around the ellipsoidal particle, where each undisturbed velocity component away from the vicinity of the particle is linearly a function of $x$, $y$ and $z$,

\begin{align*}
    u_0 &= a_0x + h_0y + g_0z + \eta_0z - \zeta_0y, \\
    v_0 &= h_0x + b_0y + f_0z + \zeta_0x - \xi_0z, \\
    w_0 &= g_0x + f_0y + c_0z + \xi_0y - \eta_0x,
\end{align*}

where all the coefficients of $x$, $y$ and $z$ are constants and the components of flow distortion and rotation. In the case of pure straining flow these equations are simplified to:

\begin{align*}
    u_0 &= a_0x, \\
    v_0 &= b_0y,
\end{align*}

(6.5)

where $a_0 = \gamma$ and $b_0 = -\gamma$, and $\gamma$ represents the strain rate of the flow.
By simplifying Jeffrey’s solution according to equation 6.5, the velocity components \( u, v \) and \( w \) (as functions of \( x, y, z, \lambda \) and the auxiliary variables in equations 6.2 and 6.3) around an ellipsoid (with the semi-principle axes of \( a, b \) and \( c \)) in the straining flow are given by:

\[
\begin{align*}
\text{(6.6)}
\text{u} &= \left[ \sqrt{\gamma + \Gamma' W - \beta' V - 2(\alpha + \beta + \Gamma)A} \right] x \\
- \frac{2\Upsilon^2}{(a^2 + \lambda)\Delta} \left[ \frac{W - 2(a^2 + \lambda)A + 2(b^2 + \lambda)B}{(b^2 + \lambda)^2} xy^2 \\
- \frac{V - 2(c^2 + \lambda)C + 2(a^2 + \lambda)A}{(c^2 + \lambda)^2} xz^2 \right], \\
\text{v} &= \left[ -\sqrt{-\gamma + \alpha' U - \Gamma' W - 2(\alpha + \beta + \Gamma)B} \right] y \\
- \frac{2\Upsilon^2}{(b^2 + \lambda)\Delta} \left[ \frac{U - 2(b^2 + \lambda)B + 2(c^2 + \lambda)C}{(c^2 + \lambda)^2} yz^2 \\
- \frac{W - 2(a^2 + \lambda)A + 2(b^2 + \lambda)B}{(a^2 + \lambda)^2} yx^2 \right], \\
\text{and} \\
\text{w} &= \left[ \sqrt{\beta' V - \alpha' U - 2(\alpha + \beta + \Gamma)C} \right] z \\
- \frac{2\Upsilon^2}{(c^2 + \lambda)\Delta} \left[ \frac{V - 2(c^2 + \lambda)C + 2(a^2 + \lambda)A}{(a^2 + \lambda)^2} zx^2 \\
- \frac{U - 2(b^2 + \lambda)B + 2(c^2 + \lambda)C}{(b^2 + \lambda)^2} zy^2 \right],
\end{align*}
\]

where \( A, B, C, U, V \) and \( W \) are given by:
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\[ A = \frac{1}{6} \left( \frac{2\alpha''_0 \gamma + \beta''_0 \gamma}{\beta''_0 \Gamma''_0 + \Gamma''_0 \alpha''_0 + \alpha''_0 \beta''_0} \right), \quad U = 2b^2 B - 2c^2 C, \]

\[ B = \frac{1}{6} \left( \frac{-2\beta''_0 \gamma - \alpha''_0 \gamma}{\beta''_0 \Gamma''_0 + \Gamma''_0 \alpha''_0 + \alpha''_0 \beta''_0} \right), \quad V = 2c^2 C - 2a^2 A, \]

\[ C = \frac{1}{6} \left( \frac{-\alpha''_0 \gamma + \beta''_0 \gamma}{\beta''_0 \Gamma''_0 + \Gamma''_0 \alpha''_0 + \alpha''_0 \beta''_0} \right), \quad W = 2a^2 A - 2b^2 B. \]

To obtain a velocity data-point located at \( x, y \) and \( z \), firstly \( \lambda \) is obtained from equation 6.1, which is a third-degree linear equation. By substituting \( \lambda \) into equation 6.2, \( \Delta \) and \( \Upsilon \) are determined. Then, all the nine integrals stated in equation 6.3 are solved numerically to compute the other ellipsoidal coordinate axillary variables. The parameters given in equation 6.9 are obtained using the axillary variables. Finally, by substituting the axillary variables (equations 6.2 and 6.3) and other parameters (equation 6.9) into equations 6.6, 6.7 and 6.8, the velocity components are determined.

6.4.3 Flow stresses over an ellipsoid in the pure straining creeping flow

In an incompressible flow, the viscous stress tensor is given by \( \tau_{ij} = \mu \left[ \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} \right] \) (using Einstein’s index notation) and the infinitesimal force \( (dF_i) \) is given by \( \tau_{ij} dA_j \), where \( dA_j \) is the infinitesimal surface. Therefore, the components of the infinitesimal force exerted on an object in the flow can be expressed as:

\[ dF_x = \tau_{xx} dA_x + \tau_{xy} dA_y + \tau_{xz} dA_z, \]

\[ dF_y = \tau_{yx} dA_x + \tau_{yy} dA_y + \tau_{yz} dA_z, \]

\[ dF_z = \tau_{zx} dA_x + \tau_{zy} dA_y + \tau_{zz} dA_z, \]

(6.10)
where \( dA_x \), \( dA_y \) and \( dA_z \) are the projection of the infinitesimal surface area of the object on \( x \), \( y \) and \( z \) planes, respectively. Using equation 6.5 the components of the infinitesimal force exerting on the infinitesimal surface area of an ellipsoid are simplified to:

\[
\begin{align*}
    dF_x &= \left[ \frac{8 \mu \Upsilon}{abc} \left( \frac{A x}{a^2} \right) - 4 \mu \Upsilon \left( \alpha_0 A + \beta_0 B + \Gamma_0 C \right) \frac{x}{a^2} \right] dA_e, \\
    dF_y &= \left[ \frac{8 \mu \Upsilon}{abc} \left( \frac{B x}{b^2} \right) - 4 \mu \Upsilon \left( \alpha_0 A + \beta_0 B + \Gamma_0 C \right) \frac{x}{b^2} \right] dA_e, \\
    dF_z &= \left[ \frac{8 \mu \Upsilon}{abc} \left( \frac{C x}{c^2} \right) - 4 \mu \Upsilon \left( \alpha_0 A + \beta_0 B + \Gamma_0 C \right) \frac{x}{c^2} \right] dA_e,
\end{align*}
\] (6.11)

where \( dA_e \) is the infinitesimal surface area of the ellipsoid, and for an ellipsoid of revolution \( (a = c \neq b) \) it is given by:

\[
dA_e = 2\pi (c \sin \theta) \sqrt{a^2 \cos^2 \theta + b^2 \sin^2 \theta} d\theta,
\] (6.12)

where \( \sqrt{a^2 \cos^2 \theta + b^2 \sin^2 \theta} d\theta \) is the infinitesimal arc length of an ellipse with the semi-principal axes of \( a \) and \( b \) shown in figure 6.5, \( c \sin \theta \) is the radius that arc is revolved and \( \theta \) varies from 0 to \( \pi \).

Figure 6.5: An elliptical path used to calculate the surface area of an ellipsoid.
Figure 6.6: An example of six overlaid LID image pairs while a filament of *A. circinalis* was trapped in the cross junction.

### 6.5 Results and discussion

Figure 6.6 shows an example of six overlaid LID image pairs captured by the PIV camera sensor while a filament of *A. circinalis* was trapped in the cross-junction. For the purpose of displaying the image, the image was post-processed and converted to a black and white image. Therefore, the particles appear larger than the real size captured by the camera sensor. Apart from the red tracer particles, images of the blue-green *A. circinalis* filament were recorded by the PIV camera sensor, in spite of the presence of the barrier filter (that blocks all the wavelengths except red) with the specifications shown in figure 6.3. This is rooted in the existence of the chlorophyll in the *A. circinalis* filament. Although chlorophyll appears green when it absorbs white light, regardless of the light source, it emits red wavelength as well (Hall *et al.*, 1993). IN the figure it is noted that
the filament is surrounded by a no particle/flow region (shown with the red dash line in figure 6.6) indicating the presence of mucilage around the filament. The mucilage was not washed off from the filament while exposed to the straining flow. Consequently, it may be concluded that those filaments with the mucilage around them do not feel the full flow stresses, and the mucilage protects them from the flow stresses. A definite conclusion will be drawn in the final chapter regarding the effect of the straining flow on the A. *circinalis* filaments.

### 6.5.1 Velocity field around the target confined in the microfluidic trap

![Image of velocity field](image_url)

**Figure 6.7:** Comparison of the ensemble-average velocity vectors (black vectors) obtained from micro-PIV measurement around a filament of the *A. circinalis* (shown in figure 6.6) and the velocity vectors (red vectors) analytically obtained from Jeffery (1922) around an ellipsoid.
The same pre-processing, processing and post-processing techniques used in chapter 4 were implemented to calculate the velocity field in the cross-slot junction of the microchannel.

To find the strain rate ($\gamma$), which is required to be substituted in the analytical solution (equations 6.6, 6.7 and 6.8), the gradients of the velocity field away from the trapped object is calculated using the same method used in section 4.11.2. The calculated strain rate was $\gamma = 3.2 \, s^{-1}$ at $Q = 50 \, \mu L/hr$, whereas in section 4.11.5, it was $\gamma = 2.3 \, s^{-1}$ at the same flow rate with the absence of the trapped filament. By substituting this value into the analytical solution, the velocity field obtained from micro-PIV and analytical solution are compared in figure 6.7. The black vectors in figure 6.7 are the ensemble-average velocity vector map in the cross junction around the trapped filament shown in figure 6.6, while the red vectors that match closely with the micro-PIV results show the analytical solution by Jeffery (1922).

**Figure 6.8:** Comparison of the $u$-component velocity profile at $y = 0$ (along the compressional axis) obtained from micro-PIV measurements and analytical solution (Jeffery, 1922) and pure straining flow.
In order to more clearly show the agreement between the PIV results and analytical solution, the $u$-component velocity profile at $y = 0$ (along the compressional axis) is plotted in figure 6.8 and also it is compared with the velocity profile when the flow was not disturbed by the trapped filament. As can be seen, the gradient of the measured velocity field is slightly higher than that of the velocity with the absence of the trapped filament ($u = \gamma x$), due to the cross-region being occupied by the trapped target, but agrees well with the analytical solutions.

6.5.2 Exerting force on a trapped object

![Graph showing the components of the force acting on the surface of the target object](image)

**Figure 6.9:** The components of the force acting on the surface of the target object shown in figure 6.6 (approximated by an ellipsoid). The exerting forces were calculated by substituting the strain rate obtained from the simultaneous micro-PIV measurement and the analytical solution by Jeffery (1922).

Using the strain rate ($\gamma$) obtained from the micro-PIV results, the components of the exerting force on the infinitesimal area of the trapped target (approximated
by an ellipsoid) were calculated using equation 6.11. The ellipsoid is stationary in the flow and therefore the resultant force acting on the total surface of the ellipsoid is zero. To find the compressional and the extensional force acting on the target we integrated equation 6.11 over one eighth of the ellipsoid surface in x, y and z directions and then multiplied it by four. In other words, we assumed that two stretching resultant forces acting along the extensional axis and two compressing forces acting along the compressional axis (schematically shown in the inset schematic of figure 6.9). The components of the force divided by the projection area at different strain rates that has a linear relation are shown in figure 6.9.

6.5.3 Estimation of the mechanical properties of an ellipsoidal object immersed in the straining flow

In our experiments we observe neither any mechanical damage to, nor any dramatic deformation in, the filaments of cyanobacteria. Therefore, at present it is challenging to obtain the mechanical properties of the cyanobacteria from the experiments. However, one can calculate the mechanical properties of an ellipsoidal object using the present experimental setup and measurement techniques if the object is deformed. The Poisson’s ratios (ν_{xy} and ν_{yx}) and the strains of the object (ε_{xx}, ε_{yy} and ε_{xy}) are determined using the deformation of the object that is imaged with the camera when the object is deformed. The stresses over the object are calculated using the method explained in sections 6.4.3 and 6.5.2. Depending on the material, one of the bidimensional models for constitutive law can be used (De Loubens et al., 2015). For example, if the material obeys the generalised Hooke’s law in a two-dimensional plane, the elasticity moduli (E_{x} and E_{y}) are determined by substitution of strains and stress of the objects into:
\[
\begin{bmatrix}
\varepsilon_{xx} \\
\varepsilon_{yy} \\
\varepsilon_{xy}
\end{bmatrix} =
\begin{bmatrix}
\frac{1}{E_x} & -\frac{\nu_{yx}}{E_y} & 0 \\
-\frac{\nu_{xy}}{E_x} & \frac{1}{E_y} & 0 \\
0 & 0 & G
\end{bmatrix}
\begin{bmatrix}
\sigma_{xx} \\
\sigma_{yy} \\
\sigma_{xy}
\end{bmatrix},
\] (6.13)

where \( G \) shear modulus.

### 6.6 Summary

We presented a new type of experiment in the microfluidic trap that enables one to measure the exerting force and the flow stresses on the trapped object using the combination of the micro-PIV results and the analytical solution of flow. In order to conduct the new experiment we combined the previous experimental setups in the preceding chapters used for micro-PIV measurements and active-control experiments. Two major imaging issues with the new setup were identified and a solution was presented.

Regarding the algae confined in the microfluidic trap, we observed a fairly large region around each alga filament, which shows the existence of the mucilage. It appears that those filaments with the mucilage are protected from the flow stresses, which to our knowledge has not been mentioned in previous studies about the effect of the fluid flow on blue-green algae.
Chapter 7

Conclusions and suggested future work

In this thesis we reported the use of fully quantified stagnation-point flows in a cross-slot type microfluidic device equipped with an advanced image-based real-time control system and simultaneously quantified by micro-PIV measurements. This enabled us to investigate the effect of the flow strain rate on the morphology of the harmful cyanobacterium *Anabaena circinalis* (also known as *Dolichospermum circinale*) by trapping their filaments in the straining flows.

In summary, in the literature there exist anecdotal evidence and sometimes reports of opposite trends regarding the mechanical damage to cyanobacteria and in particular *Anabaena* caused by small-scale turbulence. In terms of fluid mechanics measurements, previous studies relied on surrogate estimates of mean quantities of turbulence including the flow strain rate. From a microorganisms’ morphometric perspective, they judged the existence of the occurrence of mechanical damage based on measurement of the length of high throughput filaments before and after experiencing turbulence. Therefore, they neither accounted for localised strain rate of turbulence nor directly observed the possible damage to the microorganisms in real-time. This motivated us to systematically investigate the effect of the flow
strain rate on *Anabaena*, which is a common harmful filamentous, bloom-forming cyanobacteria genera in Australia. Using soft-lithography we fabricated double-layer microfluidic devices including a cross-slot type microchannel and an on-chip membrane valve. To characterise the flow and identify the uniform strain rate region, we systematically measured the velocity fields and calculated the strain rates in the cross slot junction at different flow rates using micro-PIV. Then, using an advanced image-based active control technique, we confined a large number of single filaments of *A. circinalis* at the stagnation point of the flow generated in the cross-slot junction and monitored them while they were exposed to the uniform strain rate flow. By modifying this technique we carried a novel experiment to simultaneously measure the velocity field using micro-PIV around the trapped *A. circinalis* filaments. Comparisons were also made to the stress and strain fields for an ellipsoid in an extensional flow, guided by the analytical solution of Jeffery (1922).

### 7.1 Conclusions

As the first microfluidic PhD project carried out in the Fluid Mechanics Group at the University of Melbourne, this thesis provides future students and researchers in this group with a sophisticated microfluidic experimental setup and guiding information about the microfluidic device fabrication (chapter 3), micro-PIV measurements (chapter 4), real-time control in microchannels (chapter 5) and advanced measurements (chapter 6 - simultaneous micro-PIV measurement in the actively controlled flow in the microchannel). Furthermore, carrying out different and some novel experiments has led to several important achievements and conclusions from both fluid mechanics and a microbiological perspective.

By conducting micro-PIV measurements (chapter 4) in the cross-slot type microfluidic device with no flow control, and PIV measurements in a miniature four-roll mill (appendix A) we conclude that:
Chapter 7. Conclusions and suggested future work

- Using a combination of the common averaging methods instead of a single ensemble-averaging method enabled use to study the semi time-resolved velocity field and measure the possible fluctuation of the stagnation point and simultaneously avoiding the problem of micro-PIV low image density issue.

- The cross-slot microchannel was found to be a better experimental platform than a miniature four-roll mill for the purpose of confining micron-sized objects in a controlled extensional flow. This is mainly due to the difficulty of maintaining a fixed location for stagnation point in the four-roll mill, where variations up to 50 µm were typically obtained. For the flow in the cross-slot microchannel variations were limited to 1 µm.

- Successfully carrying out the micro-PIV experiments relied on removing the bias error due to the misalignment of the camera. Omitting this error led to a reduction in the deviation from an ideal straining flow, from 4% to 2% for all of the flow rates at the central region of 0.6w × 0.6w. Therefore, the central region of 0.6w × 0.6w was identified as the uniform strain rate region with the variation of 2%.

- The strain rate and flow rate are found to be related by a linear relationship. The cross-slot channel flow was able to reach a strain rate of up to 142 s⁻¹ at a high flow rate, whereas 6 s⁻¹ was the highest reported strain rate reported in four-roll mills.

Confinement of microorganisms in the cross-slot type microfluidic device using the image-based active control system (chapter 5) has led to following conclusions:

- Using a specific combination of image processing techniques resulted in accurate detection and confinement of the filaments of A. circinalis. Due to the small size of the cell connections in A. circinalis filaments and using a camera with a low resolution (necessary to operate the active control system at a high speed), the cell connections were not detectable by the control camera.
Using a morphologically close image-processing operation by a rectangular-shaped structural element, we increased the connectivity of the blobs without over-fattening them, thereby computing the correct position of the centroid of the target.

- A large number of round motile (*Dunaliella tertiolecta*) and filamentous (*A. circinalis*) non-motile microorganisms was successfully trapped for up to an hour at the stagnation point of the controlled straining flow. Prior to the experiments we predicted the trajectory of the target object in the controlled flow. The experimentally determined trajectory of the *D. tertiolecta* agreed well with the simulation results, whereas for *A. circinalis* a discrepancy in the experimental results and numerical results was observed. The discrepancy might be rooted in the fact that in the simulation, the target object is simulated by its centroid (a point moving in the straining flow). However, the relatively large size of the *A. circinalis* and its filamentous morphology results in disrupting the hyperbolic streamlines of the flow, whereas the *D. tertiolecta*, which is much smaller than *A. circinalis* and spherical in shape, follows the flow streamlines with less influence on them.

- In contrast with previous studies that either confined micro-sized objects (e.g. Tanyeri et al. 2010) for long time scales (minutes to hours) and low strain rate (about 1 s\(^{-1}\)) or (De Loubens et al., 2015) short time scales (a few milliseconds) and high strain rate (about 500 s\(^{-1}\)), we achieved the confinement of the micron-sized objects at relatively high strain rates (up to 42 s\(^{-1}\)) for a relatively long time (up to an hour). This was conducted by programming and synchronising the pump with other equipment and gradually increasing in the flow rates using different step functions.

The last set of the experiments (chapter 6) micro-PIV measurement and real-time control were simultaneously carried out in order to measure the velocity field around a trapped target. Two major problems were encountered by combining the
two experimental steps (micro-PIV setup and microfluidic trap setup). The first issue was the disruption of the control camera by the laser pulses that prevents the detection of a target object in the micro-cross channel. The second issue was the production of extremely bright background in the images acquired by the PIV camera sensor due to the constant white light source, which precludes correlating the recorded images.

- We successfully implemented a modification to the experimental setup to address the two issues explained above. To tackle the first issue, we replaced the continuous white light with a continuous blue light source (i.e. filtering all the wavelengths except blue). Whilst, to address the second issue, we filtered all the wavelengths (except blue) hitting the control camera sensor. Using this arrangement, the control camera sensor does not capture the laser pulses, and the PIV camera does not acquire the bright background caused by the constant light source.

- Carrying out these experiments and combining the experimental results with analytical solution of flow around an ellipsoidal object immersed in a strain-inducing flow (Jeffery, 1922) enabled us to compute the flow stresses and the exerting forces on a trapped target and also visualise the flow around it.

- In the case of trapping relatively large objects such as *A. circinalis*, the average flow strain rate (obtained from the experiments) was higher than flow strain rate with the absence of the target, due to obstruction from the target object.

- In our experiment, it was challenging to obtain the mechanical properties of the trapped targets as our target objects were not deformed by the flow. However, this experiment opens new vistas of characterising mechanical properties of micron-sized objects. One can calculate the mechanical properties of an ellipsoidal object using the present experimental setup and measurement techniques if the object is deformed.
From a biological point of view the following conclusions were drawn:

- In our experiments we observed neither any mechanical damage to, nor any dramatic deformation in, the healthy filaments of *A. circinalis*, while they were exposed to a range of strain rate from 2.3 to $41.9 \text{s}^{-1}$ for up to an hour.

- Those filaments that were grown in the low-nitrate medium and had dead cells in their filaments appeared to be broken when exposed to straining flows at $\gamma = 2.3 \text{s}^{-1}$.

- It appears that filaments with a mucilaginous sheath are protected from flow stresses, which to our knowledge has not been mentioned in previous studies about the effect of fluid flow on blue-green algae. We anticipate this finding will encourage researchers to consider the presence of a mucilaginous sheath when studying the occurrence of potential mechanical damage that may be caused by flow.

- Finally, by actively monitoring the filaments under straining flow, we hold the view that the healthy filaments of *A. circinalis* are not morphologically/mechanically damaged by the strain rate of flow up to about $42 \text{s}^{-1}$, which is a relatively high strain rate compared to those that are experienced in nature. A mucilaginous sheath may play a profound role in the protection of *A. circinalis* from the flow stresses. Other investigations that reported the decrease in length of the filaments did not take the existence of the dead cells and mucilaginous sheath into their considerations.

### 7.2 Future work

From a measurement perspective, our developed experimental setup enables researchers to carry out a wide range of experiments. Our measurements and results were limited to observation of potential morphological/mechanical damage to *A.
However, using the single object manipulation integrated with simultaneous fluid mechanics measurements, one can study several other factors including growth rate, the effect of temperature on the single *A. circinalis* or other microorganisms. An example, suggested by Prof. Roman Stocker, ETH Zürich (priv. comm.), is the investigation of the Sherwood number problem (Guasto et al., 2012), (a fundamental parameter in nutrient uptake in organisms), which however, due to lack of appropriate tools has essentially never been quantified experimentally. Another example, suggested by Dr. Gursharan Chana, University of Melbourne (priv. comm.), is to conduct a combination of genomics and proteomics on *A. circinalis* to assess the proteins that change in response to the strain rate of the flow and compare with the different proteins that have changed phosphate depletion and increasing NaCl treatment reported by D’agostino et al. (2016).

The stiffness of the trap can be improved by increasing the time response of the control system using a real-time target machine with multi-core central processing unit (CPU) configurations. This will enable one to conduct the experiments at higher flow rates and strain rates, as it significantly reduce the image processing time, which is the ultimate bottleneck image-based localization system. Using stiffer trap works at high speed provides the opportunity of cell phenotyping, similar to work carried out by De Loubens et al. (2015) at longer time scales.

More importantly, one can investigate the effect of flow on the growth or destruction of the mucilaginous sheath of sheath-producer microorganisms. This requires applying higher strain rates at large time scales. Carrying out these experiments will be possible by staining the mucilaginous sheath, and observing and measuring the size of mucilaginous sheath during the experiment. Furthermore, injecting chemicals to remove mucilaginous sheath and monitoring the process can be carried out during the experiment. The presence of mucilaginous sheath producing microorganisms can cause problems for ships by increasing drag that is caused by the increase in the wall roughness when they stick to a ship hull. For submarines,
the presence of the microorganisms cause the submarine body to be detectable (due to the existence of the pigments in the cells) as well as increasing drag.
Appendix A

A miniature four-roll mill

One of the methods to produce an extensional stagnation point flow is using a four-roll mill. In this appendix we report a stand-alone experiment in a miniature four-roll mill and its feasibility to investigate the effect of the straining flow on waterborne microorganisms.

A.1 A brief introduction to four-roll mill

A four-roll mill is a device (schematically shown in figure A.1) that contains four cylindrical rollers in a fluid container. Each roller axis is set parallel to each other symmetrically in a square layout. The four-roll mill was invented by G. I. Taylor in 1934 to investigate the deformation of fluid droplets. Broadly, four-roll mills are used to study a wide range of physical phenomena. It is used to generate a broad class of two-dimensional, homogeneous flow types with linear velocity components:

\[ u = \gamma x + \omega y, \]
\[ v = -\omega x - \gamma y, \] (A.1)
where $\gamma$ and $\omega$ are the constants that are the functions of angular speed and direction of the rotation of the rollers. By a particular direction of the rotations of the rollers (shown in the top image in figure A.1), a pure extensional stagnation point flow can be produced in a four-roll mill. Here, $\omega$ is zero and the velocity equations (equation A.1) are simplified to:

$$u = \gamma x,$$

$$v = -\gamma y,$$

(A.2)

where $\gamma$ is the strain rate of the flow.

Previous works that studied the fluid flows in four-roll mills were reviewed in section 2.6.1 and it is stated that the shape of the hyperbolic streamlines (when the flow is laminar) does not change much as the angular speed of the rollers increases. It was found that the strain rate varies proportional to the cylinder frequency $f_{4rm} = 2\pi\Omega$, where $\Omega$ is the angular speed of the rollers (Andreotti
et al., 2001). In particular, the velocity gradient (strain rate) in the central region scales as $f_{4rm}$:

$$\frac{du}{dx} = -\frac{dv}{dy} = \gamma = \kappa f_{4rm}. \quad (A.3)$$

The proportionally coefficients were $\kappa = 1.4$ for a mixture of 10% glycerol in water and $\kappa = 2.7$ for the 99% glycerol (Andreotti et al., 2001). These values were approximately a quarter of that ($\kappa = 5.1$) found by Taylor (1934). However, there is no report of coefficients of proportionality for water in previous work, as water is more susceptible to instability due to its reduced viscosity compared to other fluids, such as glycerol.

The pure extensional flow becomes intrinsically unstable through a supercritical bifurcation to form an array of counter-rotating vortices aligned in the stretching direction. The Reynolds number in a four-roll mill can be defined as:

$$Re_\gamma = \frac{\gamma \Delta_{4rm}^2}{\nu}, \quad (A.4)$$

where $\Delta_{4rm}$ is the spacing between four-roll mill rollers. The critical Reynolds number is stated to be $Re_{\gamma,cr} \approx 17$ (Andreotti et al., 2001). However, the instability of the flow in the four-roll mill also depends on the other non-dimensional parameters such as the ratio of the height of the rollers to the radius of the rollers. Therefore, there is no clear evidence about the critical Reynolds number in the four-roll mills.
A.2 Experimental setup design and measurement technique

Experiments in water are crucial in our case since the algae would not survive in a medium other than water. Previous studies used large size four-roll mills with highly viscous fluids instead of water. However, we required a miniature (almost 10 times smaller) four-roll mill and with water as the medium. A miniature four-roll was designed and manufactured (shown in figure A.2) to study the feasibility of the investigation of the algae behaviour in a steady laminar extensional flow. The four-roll mill was manufactured using replaceable parts to investigate the effect of the size of the spacing between rollers alongside the size of the gap between rollers and container. A list of different configurations of the four-roll mill manufactured is presented in table A.1. These dimensions were chosen based on the critical Reynolds number \( Re_{\gamma,cr} \approx 17 \) in four-roll mills. Assuming a strain rate of about 10 s\(^{-1}\) and considering the kinematic viscosity of water \( \nu \approx 10^{-6} \text{ m/s}^2 \) resulted in the rollers spacing of 0.5 to 1 mm. To obtain the best approximation of a hyperbolic flow equation (Andreotti et al., 2001):

\[
\left( \frac{d}{2} + r \right)^2 \approx 2 \left( \frac{d}{2} + \frac{\Delta_{4rm}}{2} \right)^2 ,
\]

where \( \left( \frac{d}{2} + r \right) \) is the distance between the rollers axis and the centre of the four-roll mill, is used. The dimensions listed in table A.1 were chosen to in order to satisfy equation A.5.

Some measurements were also conducted with a different viscosity (a mixture of glycerol and water with a concentration of 99% of glycerol with the kinematic viscosity of approximately 10\(^{-3}\) m\(^2\)/s) to compare the results with other published investigations.
Appendix A. A miniature four-roll mill

(a) A photograph of the rollers of the four-roll mill  
(b) A photograph of the rollers of the four-roll mill including the container and the side windows. The walls and the rollers are painted in black to prevent the reflection of the laser light, which produce bright background in PIV recordings.

Figure A.2: Photographs of the miniature four-roll mill

Table A.1: Dimensions of the miniature four-roll in figure A.2 based on the parameters shown in figure A.1.

<table>
<thead>
<tr>
<th>Case</th>
<th>Dimensions (mm)</th>
<th>d</th>
<th>D</th>
<th>$\Delta_{4rm}$</th>
<th>$\delta$</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>22.45</td>
<td>1.5</td>
<td>0.5</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>22.45</td>
<td>0.5</td>
<td>0.5</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>28.45</td>
<td>0.5</td>
<td>3</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

Containers and rollers of varying size were manufactured and used to study the effect of the dimension of the four-roll mill on the strain rate. Four small glass windows (inside the black plastic frame in figure A.2b) were made on the cylindrical wall of the container to provide optical access from the sides of the four-roll mill.

Planar-PIV was used to measure the velocity field in the mid-section (in figure A.1) of the four-roll mill. The working fluid (MiliQ water) was seeded with one micron diameter red fluorescent polystyrene aqueous microspheres (ThermoFisher
A miniature four-roll mill

Table A.2: PIV and experimental parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image size (px)</td>
<td>$1280 \times 1024$</td>
</tr>
<tr>
<td>Zoom lens</td>
<td>VZM 1000i</td>
</tr>
<tr>
<td>Lens magnification</td>
<td>$10 \times$</td>
</tr>
<tr>
<td>Optical magnification ($\mu m$/pixel)</td>
<td>$\approx 1.5$</td>
</tr>
<tr>
<td>Depth of focus ($\mu m$)</td>
<td>50</td>
</tr>
<tr>
<td>Laser light sheet thickness $\mu m$</td>
<td>$\approx 150$</td>
</tr>
<tr>
<td>Field of view (mm $\times$ mm)</td>
<td>$1 \times 1$</td>
</tr>
<tr>
<td>Interrogation window size (px)</td>
<td>$32 \times 32$</td>
</tr>
<tr>
<td>Overlap</td>
<td>50%</td>
</tr>
<tr>
<td>Particles diameter (px)</td>
<td>$\approx 1 \mu m$</td>
</tr>
<tr>
<td>Seeding</td>
<td>Red fluorescent polystyrene particles</td>
</tr>
<tr>
<td>Flow media</td>
<td>MiliQ water</td>
</tr>
<tr>
<td></td>
<td>99% Glycerol</td>
</tr>
</tbody>
</table>

Scientific). A high speed camera with a $15.5 \times 12.3 \text{mm}^2$ CMOS sensor ($1280 \times 1024$, 10 bits, IDT Y3-Classic), that is able to capture back-to-back images at 1000 fps at full resolution, was coupled with a C-mount $2.5-10 \times$ VZM$^\text{TM}$ 1000i zoom lens to record the particle images. The minimum exposure of the camera and the pixel spacing were 1 $\mu$s and 12 $\mu$m, respectively. A 532 nm Nd:YAG double-pulsed laser (120 mJ per 5 ns pulse, CFR - BigSky Laser Series) capable of double pulsing at 10 Hz was used to illuminate the flow. The laser was operated at the lowest power and highest frequency available. A cylindrical and a spherical lens as well as an aperture (to partially block the light beam) were used to form 150 $\mu$m light sheet passing through the centre of the mid-section of the four-roll mill. All the PIV parameters are listed in table A.2 and the experimental setup and its components are shown in figure A.4.

In order to find a calibration constant to convert from pixel space to physical space, a micro-calibration target was used. The calibration constant (optical magnifications) was found to be approximately 1.5 $\mu$m/pixel. In order to mount the calibration target at the region of interest (centre of the mid-section of the four-roll mill shown in figure A.1, another container with the similar to four-roll mill were manufactured. A ring was placed in the container where the height of the ring
Figure A.3: Schematic and photograph of the calibration target placed in the centre of the mid-section of the four-roll mill.

plus calibration target were equal to half of the height of the container. Figure A.3 shows the schematic of micro-calibration.

The four-roll mill was operated at two different angular speeds 75 and 150 rpm, Reynolds number \((Re_γ)\) will be calculated after computing the strain rate. At higher angular speeds for the different cases listed in table A.1 the flow became unstable.

The same procedures in sections 4.8 and 4.9 were used to process and post-process the PIV recording. In contrary to micro-PIV, no time-averaging technique explained in section 4.7 was used to pre-process the PIV images.
Figure A.4: The planar-PIV setup to measure the velocity in the miniature four-roll mill.
A.3 Results

Figure A.5 shows an example of the velocity vector map in the 1 mm × 1 mm central region of the four-roll mill (shown in figure A.1) at the rollers angular speed of Ω = 70 rpm.

![Velocity vector map](image)

**Figure A.5:** Velocity vector map in the 1 mm × 1 mm central region of the four-roll mill (shown in figure A.1) at the rollers angular speed of Ω = 75 rpm.

The same procedure used in section 4.11.2 was implemented to calculate the strain rate and also compare the measured flow with the ideal pure straining flow. Figure A.6 shows the deviation of the flow in the four-roll mill from a pure extensional flow (equation A.2). The black and red solid lines in figure A.6 show the least square fits of the pure extensional flow equations to the values of $u$ and $v$, respectively. The error bars of the $u$-velocity and $v$-velocity data show twice the standard deviation (compressional axis) and $x$-axis (extensional axis), respectively. With the increase in the distance from the stagnation point, an increase in deviation for both velocity components from the linear profile can be seen. This deviation is rooted in the effect of rollers, disrupting the ideal extensional flow.
Appendix A. A miniature four-roll mill

Figure A.6: The solid lines represent the least square fits to the ensemble-averaged velocity and the error bars correspond to twice the standard deviation of the 95% confidence level.

The deviation from a uniform straining flow in the central region $1 \text{ mm} \times 1 \text{ mm}$ was less than 10%. In this region the relation $\frac{du}{dx} = \frac{dv}{dy} = \gamma$ is held with the accuracy of 10%.

Table A.3: Frequency and standard deviation of the strain rate and the positions of the stagnation point in the four-roll mill for different dimensions listed in table A.1.

<table>
<thead>
<tr>
<th>$\Omega$ (rpm)</th>
<th>$Re_\gamma$</th>
<th>$\sigma$ (µm)</th>
<th>$\sigma$ (µm)</th>
<th>$f_x$ (rpm)</th>
<th>$f_y$ (rpm)</th>
<th>$\gamma_{eq}$ (s$^{-1}$)</th>
<th>$\sigma_\gamma$ (s$^{-1}$)</th>
<th>$f_\gamma$ (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$x$-direction</td>
<td>$y$-direction</td>
<td>$x$-direction</td>
<td>$y$-direction</td>
<td>$x$-direction</td>
<td>$y$-direction</td>
<td>$x$-direction</td>
</tr>
<tr>
<td>Water in four-roll mill case A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>5.40</td>
<td>51.9</td>
<td>52.3</td>
<td>72</td>
<td>70</td>
<td>2.4</td>
<td>0.054</td>
<td>70</td>
</tr>
<tr>
<td>150</td>
<td>6.97</td>
<td>92.1</td>
<td>44.3</td>
<td>155</td>
<td>150</td>
<td>3.1</td>
<td>0.186</td>
<td>150</td>
</tr>
<tr>
<td>Glycerol 99% in four-roll mill case A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>0.011</td>
<td>25.8</td>
<td>34.7</td>
<td>72</td>
<td>70</td>
<td>3.2</td>
<td>0.048</td>
<td>72</td>
</tr>
<tr>
<td>150</td>
<td>0.012</td>
<td>40.2</td>
<td>69.9</td>
<td>153</td>
<td>152</td>
<td>6.1</td>
<td>0.187</td>
<td>152</td>
</tr>
<tr>
<td>Water in four-roll mill case B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>5.39</td>
<td>18</td>
<td>52.3</td>
<td>70</td>
<td>75</td>
<td>5.09</td>
<td>0.072</td>
<td>72</td>
</tr>
<tr>
<td>150</td>
<td>5.56</td>
<td>41.2</td>
<td>76.4</td>
<td>154</td>
<td>150</td>
<td>5.56</td>
<td>0.43</td>
<td>150</td>
</tr>
<tr>
<td>Water in four-roll mill case C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>75</td>
<td>0.93</td>
<td>95.4</td>
<td>78.6</td>
<td>70</td>
<td>75</td>
<td>3.7</td>
<td>0.088</td>
<td>70</td>
</tr>
</tbody>
</table>
A.3.1 Fluctuation of the stagnation point and strain rate

(a) Stagnation point fluctuations in \(x\) and \(y\)-directions at rollers angular speed of \(\Omega = 75\) rpm.

(b) Stagnation point fluctuations in \(x\) and \(y\)-directions at rollers angular speed of \(\Omega = 150\) rpm.

(c) Fluctuation of the strain rate in the central region of 1 mm \(\times\) 1 mm at both rollers angular speed of \(\Omega = 75\) and 150 rpm.

Figure A.7: Fluctuation of the stagnation point and the strain rate during seven seconds. The variation of the stagnation point was an order of magnitude of 10 microns and the variation of the strain rate was about 10%.

Similar to section 4.11.4, fluctuations of the stagnation point position and the strain rate were calculated. Figure A.7 illustrates the fluctuations of the centre and \(\gamma_{eq}\) for both roller angular speeds of \(\Omega = 75\) and 150 rpm. Frequencies (computed using FFT) and the standard deviations of the stagnation point position and the strain rate signals for all the four-roll mill cases (in table A.1) are listed table A.3. The frequencies are converted to rpm and as can be seen, the frequencies of the signals (for both cases of water and glycerol 99% as the medium) are very close to the angular speeds of the rollers. The standard deviation of stagnation
point position is an order of magnitude of 10 microns, whereas in the cross-slot microchannels they were less than one micron (shown in section 4.11.4). The maximum strain rate achieved is found to be about 3 and 6 s\(^{-1}\) for laminar flows of water and glycerol 99%, respectively. Whereas, we reached the strain rate of up to 141 s\(^{-1}\) in the microchannels (shown in section 4.11.5), and even higher strain rates could have been achieved at high flow rates. In our miniature four-roll it is possible to reach higher strain rate when using the glycerol as the medium due to the low Reynolds number. However, as mentioned above it is necessary for us to use water as the medium.

These fluctuations may stem from slight eccentric and unsynchronised rotations of the rollers. By monitoring the signal of the circuit of the rollers it was found that each roller has 3% inconsistency in the magnitude of its angular speed. Whilst to quantify the possible eccentric rotation, the rotating rollers were imaged and the locations of some arbitrary points on the edges of the rollers were tracked.

Figure A.8 shows an example of the eccentric rotation of three arbitrary points located on the edge of one of the rollers.
Appendix A. A miniature four-roll mill

A.3.1.1 Potential flow simulation

To confirm that the fluctuations of the stagnation point are caused by the eccentric rollers rotation, we simulated the flow in the whole cell of the four-roll mill by a potential flow. Eight point vortices of circulation of $\Gamma_v$ were super-positioned with the arrangement shown in figure A.9a. The inner four vortices are responsible for generating the extensional flow between the rollers, while the others simulate the wall of the container (i.e. mirror method in potential flow). The circulation of the point vortices was computed using the angular speed of rollers $\left( r\Omega = \frac{\Gamma_v}{2\pi r} \right)$. Figure A.9b shows the flow streamlines obtained from the potential flow simulation, which is similar to the flow in the whole cell of a four-roll mill.
To generate eccentric motion of the rollers, the four inner vortices were moved on a circle around their initial position. Although this type of movement is not exactly the same as the eccentric rotation of the rollers, it can estimate how the stagnation point can be affected when the rollers wobble. Applying the 3% unsynchronisation (similar to the experiment) in the magnitude angular speed of the rollers and 50 µm eccentric rotation of the four vortices of the potential flow the fluctuations in the position of the stagnation point were obtained and shown in figure A.10. The standard deviation of the signal is close to the standard deviation of the fluctuations of the stagnation point measured using PIV. Therefore, it can be deduced that the movement of the stagnation point is caused by the eccentric rotation of the rollers.

Figure A.10: The fluctuations of the stagnation point in the simulated four-roll mill obtained from the potential flow simulation.

A.3.2 Strain rate

Finally the strain rates for all three different cases of the dimensions listed in A.1 are shown in figure A.11. The results for glycerol 99% agrees with Andreotti
et al. (2001), as they used the same fluid and our four-roll mill has similar dimension ratios to their apparatus. Moreover, the figure shows that decreasing the gap between the rollers (in other words increasing the rollers radius) leads to an increase in the strain rate. This happened because of the increase in the linear velocity of the rollers and the resulting flow velocity and gradients. However, the proportionality coefficient is the same for both cases and is found to be $\kappa = 0.06$. Additionally, the single data-point for case large container shows a decrease in the strain rate compared to smaller container case.

![Figure A.11: Strain rate in four-roll mill at different rollers angular speed](image)

**A.4 Summary**

We manufactured a miniature four-roll mill (almost 10 times smaller than previous works). Using PIV the velocity field was measured and the strain rate was calculated. Lower strain rates with more vibration in the stagnation point position were obtained in the four-roll mill compared to those in the cross-slot microchannel. Miniaturisation of the four-roll mill caused small vibrations to be amplified...
when visualised with a microscope, which in turn caused fluctuations in the rate of strain. Furthermore, experiments using water is more susceptible to instability due to its reduced viscosity compared to other fluids, such as glycerol in other investigations. Fluctuations in the position of the stagnation point and the strain rate preclude confining of a microorganism in the stagnation point of the flow and study the effect of straining flow on micron-sized objects.
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