Innovative mixing technique by self-regulating pressurisation

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Abstract

This paper presents a concept of a novel sensor-controlled mixing technique. This concept provides a first feasibility study and a first gain of knowledge, whether this mixing technique can be applied in cell cultivation and similar techologies requiring soft processing conditions. at all. A microcontrol unit in combination with an electronic control board enabled synchronising several valves, a flow sensor and a liquid sensor finally allowing a pulsed flow being generated using two different sources of compressed gasses to swirl the reactor medium. The pulsation could be calibrated with the sensor-based electronics in order to ensure that volume and pressure changes during ongoing fermenter operation no longer have any influence on the pulsation. The intensity of the pulse is therefore only determined by the pressure or flow rate of the gas in the headspace or the pulsation tube. This allows to gently mix cell cultures and other in broadest terms delicate fluids.

1 Introduction

Cell cultures are increasingly used in biotechnology and in the pharmacological field, and the need for larger volumes for cell cultures continues unabated. Nevertheless, cell cultures, which are so fragile and growing so very slowly, fail to achieve the desired yield. Compared to microbial cultures, they require less oxygen due to their slow metabolism, but in spite of the demanding cultivation process, these cells are very fragile. The occurrence of shear forces - even if only due to the bursting of air bubbles - is fatal for these organisms. The agitation of the medium to provide these cells with fresh medium becomes a real challenge with increasing volumes. Substrates and other nutrients have to be supplied and metabolites have to be dissipated, so a homogeneous circulation of the medium is absolutely necessary.

1.1 Actual situation

The cultivation of human and animal cells is still mostly conducted in cell culture flasks, whose volume is very limited. The advantage of these flasks, besides their single-use principle, is the large surface area of the medium offered. This extended surface area is necessary because these cell culture flasks allow a sufficient exchange of gases for the cells. The bottles do not contain any stirrer, additional gas supply or any control (e.g. for pH or O_2).
In the case of increased volumes and for pH / oxygen control or the addition of nutrients, larger systems such as fermenters / bioreactors are required. These vessels are made of stainless steel or borosilicate glass and are stirred while regulated by a bioreactor control system and supplied with all necessary media.
With these very expensive systems, microorganisms such as E.coli can be produced fast and efficiently to high cell concentrations, in order to produce their biomass or to obtain higher-value substances such as proteins. The situation is different for cell cultures - they have also a very high potential to produce, for example, valuable drugs/proteins. But while the growth rate of microbial organisms is within a half-hour range, cell cultures require several (15 - 25) hours [1].
Previous studies conducted in the 1980s [2] showed how sensitive these cell cultures are when exposed to shear forces for a longer period of time.
Shear force-sensitive means that the cells are severely affected by larger pressure fluctuations/changes. These can be induced, for example, by fast-moving stirrer blades.
For this reason, the process has been performed in classical cell culture flasks for as long as possible.
Bioreactors with agitator shafts and special impeller shapes are used to homogenize the medium even on a larger scale. The aeration is usually performed by microspargers - to produce small bubbles that prevent inducing cavities that cause the cell to burst. There are special stirrer types for cell cultivation, such as 3-blade segment stirrers - these must not exceed a speed of 300 rpm to avoid causing damage to the cells. Especially in cell culture, where cell-cell communication takes a major role, the destruction of a few cells can already initiate a signal cascade.
Cells that are still living may be affected or even start their automated cell death (apoptosis). Homogeneous dispersion of cell culture media in larger scales remains challenging.
In the centre of a bioreactor an agitator shaft with impellers is located - a 2L stirred reactor Univessel® for example has an inner diameter of 130mm. Within this area, all built-in components such as pH and oxygen probes, exhaust air coolers, addition nozzles, etc. are required. A large area is taken up by the agitator. They are usually manufactured with a size ratio of 3:1 (reactor inner diameter : stirrer diameter). This means that (i) a large area of the reactor lid is used only for the agitator shaft (ii) the cost of such agitator shafts including impellers costs around €1,500 (iii)

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the stirring movement of the agitator shaft deposits abrasive particles through the mechanical seal and (iv) this drive technology causes additional costs due to the frequent replacement of wear parts.

1.2 Pulsation as an innovative approach

In this article, a well-known but not yet established mixing technique is presented, which has been further improved with sensor-based electronics.
The above-described shear force problem of cell cultures could be solved by this innovative mixing technique. The so-called pulsation, where in the middle of the fermenter a pulsation tube has been installed. The medium in the fermenter will be pulsed up and down with an overpressure swing technique. These mixing technologies are used daily in our laboratories, e.g. to mix biological material using pipettes. The same process can also be used for cell cultures in fermenters / bioreactors - and finally for low-cost bioreactors such as Schott bottles.
This pressurized mixing technique is a well-known technology which has already been described in several patents, but it has not been able to gain proper acceptance yet. In earlier pulsation arrangements, wherein the gas was pulsed into a tube, the system could swing up (the medium is displaced more and more and finally also pressed into the sterile filter, causing blockage). This effect can result in an unstable process or even product failure and may be caused even by the slightest pressure fluctuations.

1.3 Construction

The pulsation tube with a diameter of 6 mm is mounted in the centre of the fermenter - just like the classic agitator shaft - but can be installed in a classic Univessel® port as for level probes with a 6 mm clamp connector. In this project, an adapter was manufactured (**Fig. 1**), which can be installed in the bioreactor lid replacing the agitator shaft. The pulsation tube itself can be fixed in a height-adjustable configuration with a union nut and clamping cone. The centre of the bioreactor is ideal, as it ensures the optimum position for dispersing the medium downwards in all directions. However, other positions of the pulsation tube are still conceivable / possible.
A reactor lid needs at least three ports (i) an inlet for the aeration of the headspace to generate an overpressure (ii) an outlet for the exhaust air (bioreactors have an exhaust cooler) and (iii) a port for the pulsation tube itself - ideally with a clamping connector as described above to be able to adjust the insertion depth of the pulsation tube. For the first experiments, a Schott bottle with a 3D-printed head piece and a bored lid was used as a simple experimental setup (**Fig. 2** – see next page).
A 500ml Schott bottle was used as a low-cost bioreactor. This type of bottle has a standardized GL45 thread. Thus, this setup can be applied to all Schott bottles of the sizes 50 ml to 20 L - only the length of the pulsation tube has to be adapted. Inside the bottle, Lewatit® ion exchange resin in spherical form was used to illustrate the fluidic motion - these small (0.62mm) beads have a density of 1.06g/cm³ and can successfully visualize the turbulence in the bottle. This made it possible to evaluate whether this setup (**Fig. 3** – see next page) could be implemented at all and whether the medium could exhibit the desired behaviour.

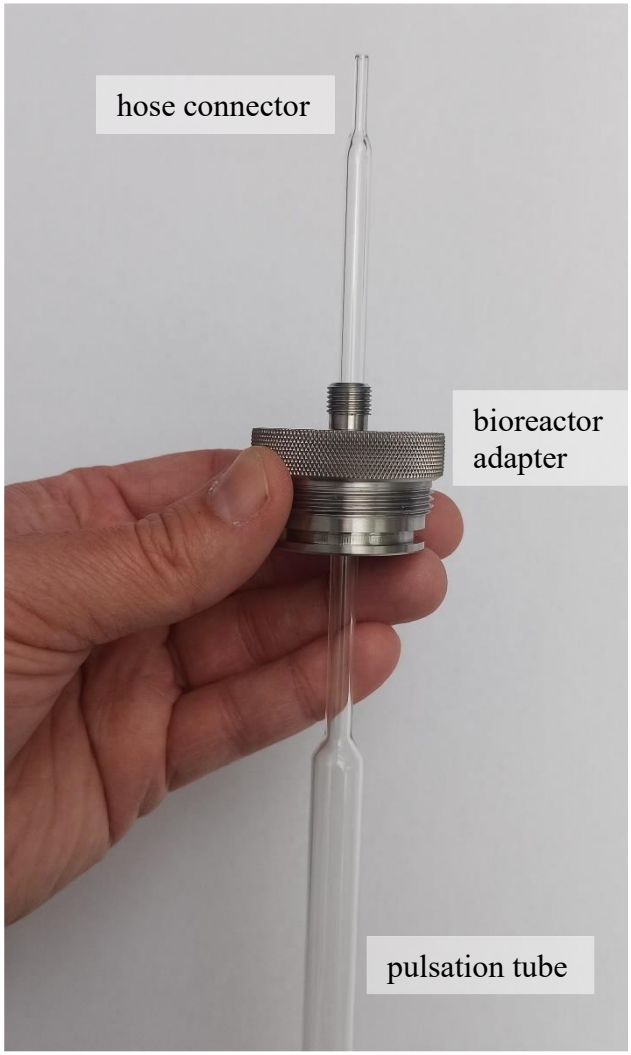

Figure 1 Adapter for height-adjustable pulsation tube (12x1 OD x WS). The hose nozzle at the top end turns into a 6x1 tube piece in the adapter for Univessel® Ports and is clamped there (this is normally used for agitation shafts).

Particular attention was paid to a swing-up of the system during longer test periods. For the current test, no special installation such as an aeration hose or microsparger was used to create overpressure in the headspace. However, these installations are possible if bubble-free aeration is necessary or if the oxygen is to be added submerged.
The gas supply for the bioreactors is later to be controlled by a stand-alone system. In this bioreactor control, a specific gas composition or gas mixing ratio and flow rate is required for the application. Depending on the equipment, such a bioreactor control provides at least 2 different gas outlets (headspace and submers). In the headspace mostly CO_2/Air mixture is used and O_2/N_2 mixture is added submerged e.g. via a microsparger or a thin-walled aeration

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tube. Other gas mixtures are also possible and not relevant for such a mixing technique. The mixing technology as described here aims at retrofitting old or existing systems with such an interconnected pulsation system. The described gas supply system can also operate with a single gas mix, which, however, would have to be divided into two paths.

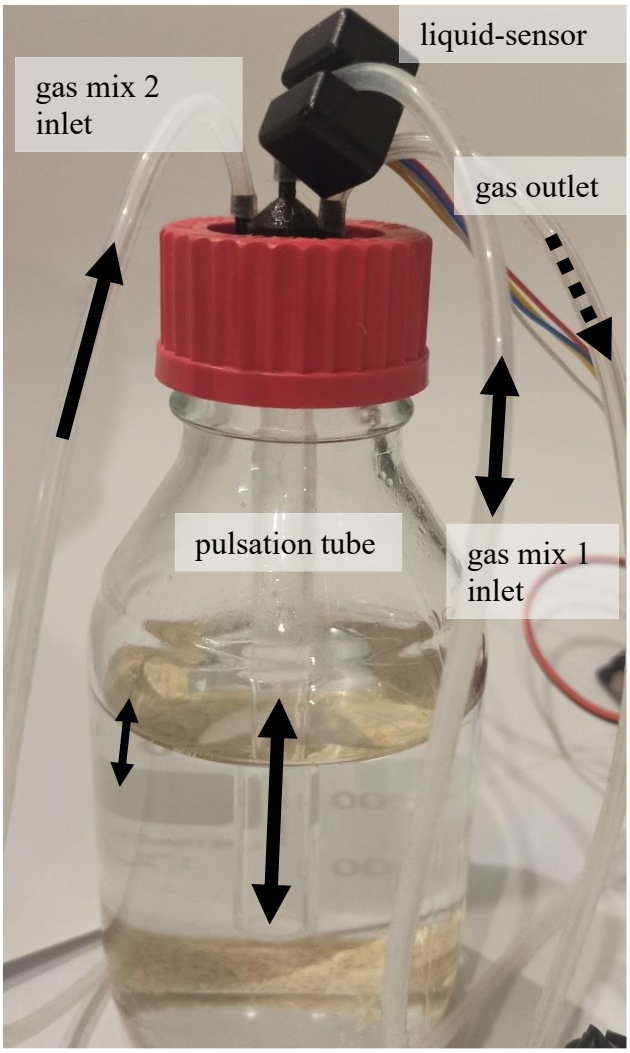

Figure 2 500ml Schott bottle with a 3D-printed headpiece for the gas supply. The pulsation tube was placed in the centre. The arrows mark the direction of the gas flow or the movement of the medium.

The setup of the system can be described as follows: The two gas outlets (gas mix 1 and 2) of the bioreactor control system are connected via PU hoses to the pulsation system, where gas mix 1 - with which pulsation is to be performed - is channelled via a flow sensor (Sensirion - SFM4100). The second gas (gas mix 2) from the bioreactor control (usually intended by the user for submers) can optionally(!) be led into the bioreactor via an aeration tube or microsparger - however, shear forces could be induced by the small bubbles. Alternatively, the gas mix 2 can also be fed into the headspace. The gas mix 1 flows through the flow sensor, which measures the flow rate of the gas into the fermenter.

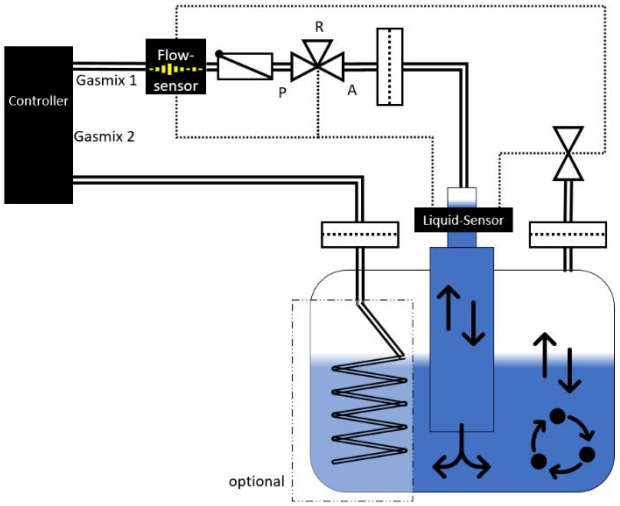

Figure 3 Schematic set-up for the pulsation movement. The use of aeration hoses is optional. The medium in the glass vessel is moved up and down by the pressurised air from the bioreactor control system. A flow sensor and a liquid sensor are used to stabilise the fluid movement. The valves for overpressure control are switched by these two sensors.

Standard bioreactor controller use a MFC (mass flow controller) for gas supply, therefore the flow should be constant for both gas mix 1 and 2. Gas mix 1 is further led via a check valve and passes to a 3/2-way valve (Bürkert - direct acting type 6012). The gas flows into the inlet side (P - normally closed) of the valve and, as the medium is pushed back into the reactor vessel, flows to the outlet side (A). The second output (R) is connected normally open between A and R.

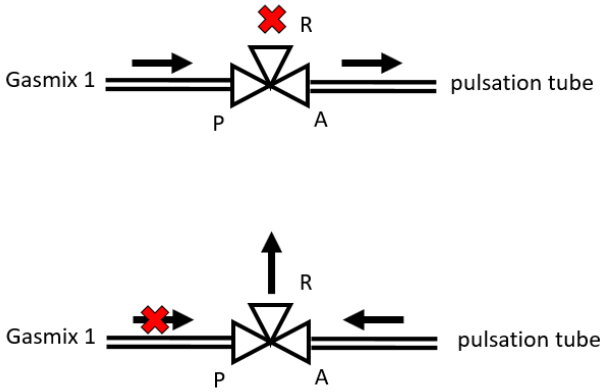

Figure 4 Schematic illustration of the operation of the 3/2-way valve (normally closed - T-universal) for pulsing the gas mix 1.

The output side (A) is fitted with a silicone hose (with a sterile filter in between) which is passed on to a liquid sensor and subsequently to the hose nozzle of the pulsation tube. The liquid sensor works contact-free via ultrasound and can detect within a few milliseconds whether there is

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medium (regardless of density, temperature or pressure) in the hose. The gas mix 1 pushes the medium - which is in the pulsation tube - back into the vessel. During this time, the flow sensor measures the volume of the gas that passes through gas mix 1 by counting the flow rate. During initial installation, the user only has to set the maximum internal volume of the pulsation tube. When the defined volume is reached, the 3/2-way valve switches over so that the gas mix 1 in the pulsation tube can return to the second outlet side (R) of the 3/2-way valve via the pulsation tube (**Fig. 4** – see previous page).

The gas mix 2 flows at a constant speed into the headspace of the bioreactor and generates an overpressure. This headspace overpressure ensures that the medium is pressed back into the pulsation tube (as soon as the 3/2-way valve is in the open position A-R). To prevent the overpressure in the head space from escaping via the exhaust cooler, a 2/2-way valve is installed behind the exhaust cooler, which remains closed while the 3/2-way valve is in the A-R position. The gas in the pulsation tube can be vented via the 3/2-way valve. As soon as the medium in the pulsation tube is pressed further up to the silicone tube / liquid sensor, the liquid sensor transmits the signal that the 3/2-way valve switches to P-A position (so that new gas is pressed into the pulsation tube and thus into the reactor). At the same time, the 2/2-way valve located at the exhaust cooler is now opened, the overpressure in the headspace can be released via the exhaust cooler. This causes the medium in the silicone hose and pulsation tube to be pressed back into the bioreactor vessel.

The press-back of the gas mix 1 is carried out via the flow sensor, which pushes the gas into the pulsation tube with exact volume. This process can be repeated as often as required and at various pressures. The speed of the medium being pressed up into the pulsation tube is determined by the pressure / flow rate in the headspace or by gas mix 2. The speed of the medium being pushed back from the pulsation tube into the reactor is determined by the pressure / flow rate of gas mix 1. Depending on the features of the bioreactor controller, this can be a few millilitres to a several litres per minute - for glass vessels, the allowed pressures range up to 1 bar (up to 3 bar for in-situ systems). The valves and sensors used are approved for up to 6 bar.

Both gas mix inlets may have different pressures as well as flow rates (cf. **Fig. 5 and Fig. 6**). The pulse rate during pulsation can be as short as 10 ms per pulse - depending on the kind of 3/2-way valve being used.

Tests revealed that during the pulsation, the headspace was pressurized below 100 mbar. The speed of the medium in the pulsation tube depends only on the pressure and flow rate of the two gas mixes 1 and 2 released by the bioreactor controller.

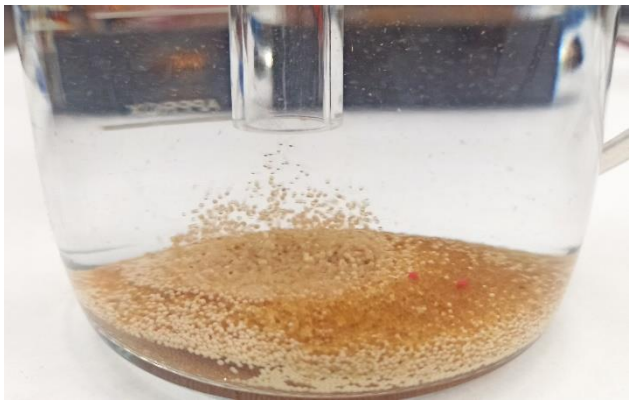

Figure 5 Pulsation with low pressure. A slight turbulence of the Lewatit® beads is visible. The inner volume of the pulsation tube was approx. 5 ml.

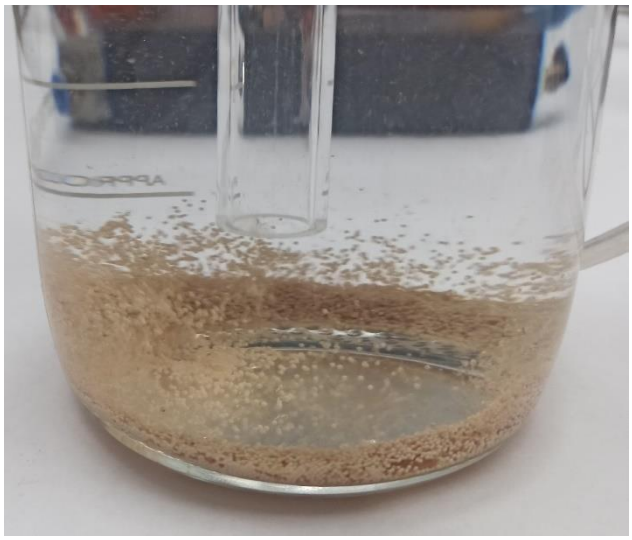

Figure 6 Pulsation with higher pressure but the same pulsation volume of 5ml. The vortexing of the Lewatit® beads is much stronger. Such strong turbulence is probably not suitable for cell cultivation.

The gas supply was tested with two different membrane pumps from the aquaristic application in order to simulate different pressures and flow rates before use in bioreactors. The program sequence provides first of all the pulsation tube is filled with medium, i.e. the 2/2 way valve connected behind the exhaust cooler is closed and the pressure at the headspace increases until the liquid sensor receives a signal. After this, gas mix 1 is pressed back into the pulsation tube and therefore also the medium into the fermenter.

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The pseudo code can be described as follows:

```
step 1
3/2-way valve = 0;
2/2-way valve = 0;
if liquid-sensor = 1
x = 0ml;
        step = 2;

step 2
3/2-way valve = 1;
2/2-way valve = 1;

if x > volume pulsation pipe
        step 1;
else
x = x + (flow/time);
```

The Arduino Uno toggles between these two states. In the current setup, the Arduino is still powered via the USB interface - also for the reason the pulsation volume can be changed online via the same interface. The first test setup revealed a weak point: if the gas mix 1 was not connected, the pressure in the headspace caused medium to be pressed all the way into the flow sensor. For safety reasons, a check valve has been installed between the flow sensor and the 3/2-way valve. For the future, a program-based solution will be developed to interrogate the flow rate and flow direction. The main problem with this project is currently the reaction rate of the programming.
The system was implemented on an Arduino Uno, which can control both valves and both sensors through a controller board. However, the Arduino Uno is not fast enough to be able to control all processes, such as the acquisition of the signals, the recalculation of the flow rate and the toggling of the valves fast enough - especially when there are pressure fluctuations. This is more a matter of optimizing the programming or transferring it to a more powerful system such as the Raspberry Pi. For first laboratory applications the Arduino Uno will be sufficient, since it is not to be expected that the pressure conditions change strongly while running the system. For everyday laboratory use, gas mix 1 and 2 are maintained at a constant flow rate via MFC in the bioreactor control system.
When the oxygen consumption of the cells increases, the oxygen level is raised via the gas mix even before the flow rate is further enhanced itself. Hence, the first prototype proved to be a successful system, which currently only has a weakness in programming performance and will be transferred to a more powerful system within the next weeks. In addition, the first realistic tests will be conducted in a cell culture laboratory, comparing a stirred and a pulsed cell culture.

One of the biggest advantages is the stability of the system (**Fig. 7**) even if additional exposed gases are generated during the cultivation (e.g. by the microorganisms themselves). Also, the system is not affected by further addition of fluids during operation, which only results in a rise of the level in the reactor vessel. One of the key points is the safety of the system, even if the filter at the exhaust cooler gets clogged over the time (e.g. due to condensation in the filter), this mixing technique (i) will inform the user about the irregularity / interruption of the pulsation cycle of the liquid sensor and (ii) if the flow rate should change as a result of the aeration strategy, the flow sensor will automatically compensate and change the valve timing so the gas volume in the pulsation tube can continue to flow through at a constant rate. Therefore, the system enables a self-regulating process with sensor-assisted pressurization and coupled flow sensor technology - an application in extended dimensions is highly possible.

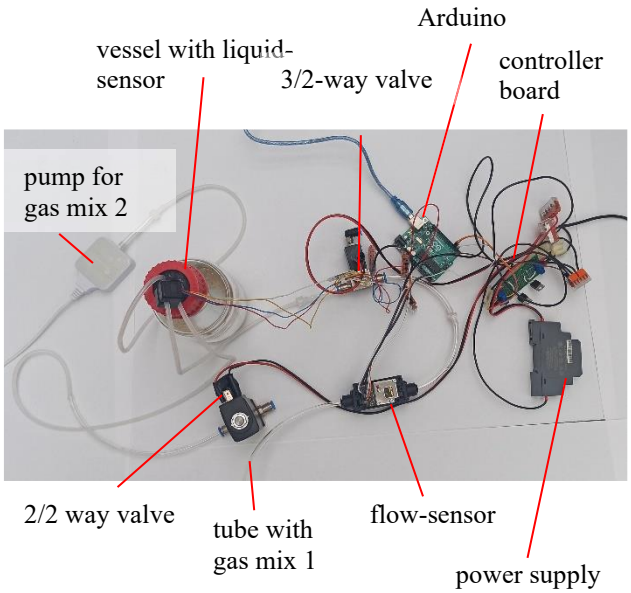

Figure 7 The current set-up for the proof-of-principle. All components will be installed in a housing for real use in the laboratory. For the user, only the gas inlets and outlets and the sockets for the power supply will be visible.

2 Literature

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