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LONG-TERM WATER QUALITY DATA EXPLAIN INTER-POPULATION VARIATION IN RESPONSIVENESS TO STRESS IN STICKLEBACKS AT BOTH WASTEWATER EFFLUENT-CONTAMINATED AND UNCONTAMINATED SITES

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Abstract

The magnitude of the corticosteroid response to a standardised stressor varied in proportion to the concentration of effluent in three-spined sticklebacks (*Gasterosteus aculeatus* L.) captured downstream of 10 wastewater treatment works (WWTW). However, at 9 sites with no upstream WWTW input inter-population variation in the reactivity of the stress axis occurred across a similar range to that seen for fish at impacted sites, suggesting that the factor(s) responsible for modulating stress responsiveness in sticklebacks are not unique to sites receiving WWTW effluent. Physicochemical data from a long-term monitoring programme were employed to investigate whether variation in water quality contributed to between-site variation in stress axis reactivity. Between-site variation in fourteen water quality determinands explained between 30% and 60% of the variation in stress reactivity, and fish size, for sticklebacks at both WWTW-contaminated and uncontaminated sites. At uncontaminated sites the mean mass and length of sticklebacks increased with total oxidised N concentration (as an indicator of anthropogenic input) whereas at WWTW-contaminated sites fish size decreased with increasing effluent concentration, suggesting that factors adversely affecting growth were present predominantly at WWTW-contaminated sites. In contrast, at contaminated and uncontaminated sites the magnitude of the corticosteroid response to a standardised stressor increased with anthropogenic input (effluent concentration or total oxidised N respectively), indicating that factor(s) modulating the reactivity of the stress axis may be present at both WWTW-contaminated and uncontaminated sites.

Keywords: Stress response, Wildlife toxicology, Water quality, Municipal effluents, Cortisol, Stickleback.

INTRODUCTION

It is well established that chemicals within the aquatic environment can cause adverse effects in wildlife by interfering with the normal function of endocrine-dependent processes [1,2]. Most research into the effects of endocrine-modulating chemicals on aquatic vertebrates during the past two decades has focused upon disruption of the reproductive endocrine axis [3]. Much less attention has been directed towards contaminant-related modulation of non-reproductive endocrine processes and in particular the hypothalamic-pituitary-adrenal/interrenal axis (HPA/I axis) [4,5] which plays a key role in responding to biotic and abiotic challenges in all vertebrates. The HPA/I axis underpins the neuroendocrine stress response and as such is part of a suite of adaptive responses, the functional integrity of which is of great importance to the individual [6]. Biota within the aquatic ecosystem are particularly vulnerable to chemical exposure given the range and quantity of anthropogenic contaminants that are introduced into watercourses. A substantial proportion of those contaminants originate from wastewater treatment works (WWTW) [7] whose effluents can comprise a significant proportion of the total flow in receiving waters [8].

The stress axis in fish is known to be adversely affected by exposure to metals [9,10,11,12] and organic chemicals [13,14,15]. Recent studies in rainbow trout *Oncorhynchus mykiss* Walbaum [16] and the three-spined stickleback *Gasterosteus aculeatus* L. [17, 18, 19] have suggested that WWTW effluents may contain chemicals that affect the magnitude of the response mounted by the fish to stressors, a finding with clear implications for the ability of individual fish to respond to challenging events. In sticklebacks at sites affected by upstream WWTW discharges, the magnitude of the cortisol response to a standardised stressor is broadly proportional to the effluent concentration to which each population is exposed [17, 19]. However, among sticklebacks at sites with no identifiable upstream WWTW inputs significant between-population variation in the magnitude of the stress response to a standardised stressor is also evident (T. G. Pottinger, unpublished) and encompasses the range of responses seen among fish at WWTW-contaminated sites. This suggests that if inter-population variation in stress axis function is a consequence of chemical exposure, then the factors responsible, although they vary in proportion with WWTW effluent input, are not uniquely associated with WWTW effluents.

As a first step to identifying candidate causal factors we examined the variation in stress axis function among populations of sticklebacks at both WWTW-contaminated and uncontaminated sites in relation to a range of basic water quality indices. By this approach we sought to establish whether variation in stress axis reactivity among fish at both types of site was related to variation among the physicochemical profiles at these sites. In doing so, we made the assumption that the suite of determinands widely used to define water quality would reflect anthropogenic inputs from a wide range of sources (both point and diffuse) and would thus provide a proxy index for chemicals from the same sources likely to co-vary with the primary determinands. Because the sampling and processing of blood samples from small fish at remote field sites is difficult to accomplish we quantified the magnitude of the stress response in the target species by measuring the rate of release of cortisol across the gills. Cortisol release to water by fish has been shown to be an effective surrogate for blood cortisol levels during stress [20] and is a more consistent indicator of WWTW-related effects than the alternative approach of determining whole-body concentrations of cortisol [19].

METHODS

Site selection and sampling procedure

Three-spined sticklebacks were collected from nine sites on rivers within the Lancashire, Cheshire, Merseyside and Greater Manchester area of north-west England at which no upstream WWTW inputs could be identified (Table 1) and from ten locations downstream of the effluent discharge points of wastewater treatment works (WWTWs; population range 22000 - 120000; Table 2) in the same geographical area. Approximately 20 three-spined sticklebacks were captured at each site during March 2014 using a metal-framed 45 cm D-profile hand net. After capture, ten fish were retained in a bucket containing river water for a period of 30 - 45 minutes before being transferred to individual capped Nalgene tubs (150 mL, 6.5 cm diameter) each containing 100 mL artificial freshwater (deionised water, 0.33 g/L aquarium grade sea salt) [21]. The fish were retained in the tubs for a further 30 minutes in order to collect stress-induced cortisol released to water (CRTW). Because of the constraints associated with sampling in rivers at remote sites there was unavoidable variation in the time that elapsed between capture and termination. However, in three-spined sticklebacks concentrations of both plasma cortisol and whole-body cortisol (and by inference CRTW) reach a stable plateau within 30 minutes of first exposure to an ongoing stressor. This plateau is sustained for at least an additional 30 – 60 minutes ([22]; T. G. Pottinger, unpublished data). The remaining fish were killed immediately and processed as described below. Artificial freshwater, rather than the river water at each site, was used for the collection of CRTW to minimise the inclusion of suspended solids likely to interfere with the subsequent extraction procedure, and to allow the collecting vessels to be prepared in advance. However, we don't believe this substantively affected the rate of cortisol release observed. After the confinement period each fish was killed by immersion in a high concentration of sedative (2-phenoxyethanol, 1:1000) and transferred to individual labelled 12 mL capped polypropylene test tubes which were placed in a dry shipper (Taylor-Wharton CX500) for transport back to CEH Lancaster where the samples were stored at -70^{o}c prior to processing. The collection vessels containing water samples were held on ice in coolboxes until return to CEH Lancaster where they were transferred to a freezer (-20^{o}C) for storage. The confinement stress procedure was approved by the Lancaster University Animal Welfare and Ethical Review Body and was conducted under Home Office licence. In the laboratory, each fish was weighed to the nearest mg, total length was recorded to the nearest mm and the sex of each fish was determined, after making a ventral incision, by macroscopic examination of the gonads. The coefficient of condition (K, Fulton's condition index; [23]) was calculated as K = (100 x weight)/(length^3).

Extraction of water samples for cortisol determination

Water samples within which single sticklebacks had been held post-capture for a period of 30 mins were thawed at room temperature. Each sample was pumped (Watson Marlow 202S multi-channel peristaltic pump, 10-20 mL/min, 12 active channels, 2.79 mm i.d. silicone tubing) through an inline 0.45 µm pre-filter (Pall Gellman Acrocap, Pall Life Sciences) and a Sep-Pak C18 cartridge (Waters Ltd). Sep-Pak cartridges were cleaned and conditioned by flushing with 5 mL of ethyl acetate, followed by 5 mL methanol and 5 mL deionised water in a vacuum manifold. The cartridges were not allowed to dry out between conditioning and receiving the water sample. One blank (100 mL artificial freshwater only) and one recovery standard (100 mL artificial freshwater containing a 100 µL aliquot of a solution of cortisol in ethanol, 5 ng/mL) were included with each batch of ten water samples (100 mL). No interference was detected in any of the samples and recovery of added cortisol was consistently >85%. After extraction, cortisol was immediately eluted from the Sep-Pak cartridge in a vacuum manifold with 2.5 mL ethyl acetate. The eluate was dried in a heating block under a stream of air at 40^{o}C and redissolved in 350 µL ethyl acetate. A 150 µL aliquot of the reconstituted extract was taken for assay.

Cortisol radioimmunoassay

A previously validated method [24] was employed to analyse cortisol concentrations in water extracts, with two minor adjustments. The antibody used in this study was IgG-F-2 rabbit anti-cortisol (IgG Corp; Nashville, TN, USA) and tracer ([1,2,6,7]^{3}H-cortisol, 2.59 TBq/mmol; Perkin-Elmer, U.K.) was added in a 25 µL aliquot of buffer at the same time as the antibody was dispensed.

Estimation of effluent concentration at WWTW-contaminated sites

The percentage of WWTW-derived effluent at each sampling site was estimated using the Low Flows 2000 Water Quality eXtension model (LF2000-WQX model). The LF2000-WQX software combines hydrological models with water-quality models to make predictions on the concentration of a given chemical originating from a point source, such as WWTWs [17,25]. The estimated WWTW effluent concentrations are shown in Table 2.

Water quality data

The UK Environment Agency Water Information Management System (WIMS) water quality data set was accessed to provide chemical data for locations close to sites from which sticklebacks had been sampled (WIMS sample site locations are shown in Tables 1 and 2). The physico-chemical water quality determinands retrieved from the database comprised: dissolved O_2 (mg/L), water temperature (^oC), pH, suspended solids (mg/L), total oxidised N (mg/L), NO_3 as N (mg/L), NO_2 as N (mg/L), NH_3 as N (mg/L), un-ionised NH_3 as N (mg/L), reactive orthophosphate (total reactive phosphorus), as P (mg/L), reactive Si, as SiO_2 (mg/L), Mg (mg/L), Cl (mg/L), Zn (µg/L), and Cu (µg/L). The mean number of samples per determinand was 251 (median = 216) excluding the metalloid Si and metals Mg, Cu and Zn for which the number of samples was more limited (mean = 108; median = 45). The mean time period across which samples were collected for the two groups was 22 years and 12 years respectively. Many of the water quality variables exhibited cyclical seasonal variation, however, in order to simplify analysis the assumption was made that underlying between-site differences in the concentration of determinands would be reflected in the overall mean value for each site. The means for water quality data at sites downstream of known WWTW discharges displayed trends consistent with those predicted and we therefore concluded that adopting this approach to collating the data was fit for purpose.

Statistical analysis

The effect of an upstream WWTW discharge on variation among the water quality data, and the extent of between-site variation in water quality, were assessed using a general linear model (GLM; Minitab 16; Minitab Inc.) with sample site nested within pollution status (presence or absence of upstream WWTW) and post hoc pairwise comparisons of means by Tukey's method. The relationships between water quality determinands at each site and somatic and endocrine parameters in sticklebacks at those sites were investigated using multiple linear regression (Minitab). Trends within somatic and endocrine data ranked by either the total oxidised N concentration at each site (uncontaminated sites) or by site effluent concentration (WWTW-contaminated sites) were assessed by Spearman's rank order correlation (Sigmaplot v. 13; Systat Software Inc.) and graphically depicted using linear regression (Minitab).

RESULTS

Differences in physicochemical indicators of water quality between WWTW-contaminated and uncontaminated sites

The presence of an upstream WWTW discharge was a significant determinant of variation in all the chemical data examined with the exception of suspended solids, unionised NH_3, Cl and dissolved Cu which did not vary significantly between sites with or without an upstream WWTW discharge (Table 3). The greatest differences in overall mean values for water quality variables between WWTW-contaminated and uncontaminated sites (the term "uncontaminated" is used throughout to denote sites not affected by upstream point source WWTW effluents) were seen for NO_3, NO_2, orthophosphate, Si and Zn which in all cases were significantly higher at sites downstream of WWTW discharges than at sites with no known upstream WWTW inputs (Table 3). All water quality variables exhibited significant between-site variation (GLM: $p < 0.001$) and despite the overall effect of upstream WWTW discharges there was considerable overlap in the range of site-specific means between WWTW-contaminated and uncontaminated sites. To illustrate this, the data for total oxidised N ($NO_3 + NO_2$) and orthophosphate from all sites are shown in Figure 1. Mean values for all the determinands at each site are presented in the Supplemental material (Supplemental Data, Table S1). For sites uncontaminated by WWTW effluent the individual site data are ranked by total N concentration (the quantitatively dominant water quality indicator) and for WWTW-contaminated the sites are ranked by estimated effluent concentration (the defining characteristic of these sites).

Trends in size and stress responsiveness related to water chemistry and effluent concentration among sticklebacks at WWTW-contaminated and uncontaminated sites

There was no significant difference in size overall between male and female fish at uncontaminated sites in terms of mass (ANOVA, $F(1,170) = 0.0$, $p = 0.98$), length ($F(1,170) = 0.0$, $p = 0.87$) or condition ($F(1,170) = 0.1$, $p = 0.82$). At WWTW-contaminated sites there was no difference between the sexes in terms of length ($F(1,190) = 2.9$, $p = 0.09$) or condition ($F(1,190) = 1.5$, $p = 0.2$) but females were significantly heavier than males ($F(1,190) = 6.72$, $p = 0.01$). Fish were significantly heavier ($F(1,362) = 53$, $p < 0.001$) and longer ($F(1,362) = 53$, $p < 0.001$) overall at WWTW-contaminated sites than uncontaminated sites but there was considerable overlap in the range of site means for each group (Figure 2). Among fish from uncontaminated sites mean mass and length increased significantly with increasing mean concentrations of total N at the sample location (Figures 2a and 2b; mass: Spearman's correlation coefficient = 0.40, $p < 0.001$; length: Spearman's correlation coefficient = 0.45, $p < 0.001$). At two sites, (Old Eea Brook and Town Brook) the mean mass and length of sampled sticklebacks was considerably greater than that of fish at other uncontaminated sites ($p < 0.05$; Tukey's test), these sites were omitted from the correlation and regression analyses. At WWTW-contaminated sites, there was a negative trend in both mass and length in relation to increasing effluent concentration (Figures 2a and 2b; mass: Spearman's correlation coefficient = -0.38, $p <$ 001; length: Spearman's correlation coefficient = -0.39, $p < 0.001$). The coefficient of condition (K) did not vary significantly with either total N or effluent concentration, for either sex (Spearman's correlation coefficient < -0.15, p > 0.14; Supplemental Data, Figure S1). A summary of all the somatic data is presented in the Supplemental Data (Tables S2 and S3).

The stress-induced rate of release of cortisol was significantly greater overall for females (1736 ± 151 pg/g/h, n = 105; Figure 3a) than males (818 ± 92 pg/g/h, n = 81; $F(1,184) = 23.5$, $p < 0.001$; Figure 3b) and there were significant trends in CRTW which at uncontaminated sites increased with total N (Figure 3a: females, Spearman's correlation coefficient = 0.56, $p < 0.001$; Figure 3b: males, Spearman's correlation coefficient = 0.57, $p < 0.001$) and at WWTW-contaminated sites increased with effluent concentration (Figure 3a: females, Spearman's correlation coefficient = 0.36, $p < 0.01$; Figure 3b: males, Spearman's correlation coefficient = 0.43, $p < 0.01$).

The relationship between indices of water quality and somatic and endocrine parameters

The outcomes of multiple regressions conducted between the array of water quality indices for each site and the somatic and endocrine data for fish captured at those sites is shown in Table 4. Seven water quality analytes (NO_2, un-ionised NH_3, suspended solids, Cl, orthophosphate, reactive Si) were excluded from the regression equations for non-WWTW sites because they were highly correlated with other factors. The same group of analytes together with NO_3 were excluded from the WWTW site regressions. The regressions suggest that 50-60% of variation in weight and length at both WWTW-contaminated and uncontaminated sites variation can be explained by variation in water quality (Table 4). At both WWTW-contaminated and uncontaminated sites a substantial proportion of the variation in stress-induced water cortisol release was explained by variation in water quality (27-45%; Table 4).

DISCUSSION

The neuroendocrine stress response is a key adaptive mechanism that allows animals to cope with adverse changes in their environment and to respond appropriately to a wide range of threats and challenges [6]. Recent observations have suggested that prolonged exposure to WWTW effluent modulates the magnitude of the corticosteroidogenic stress response in three-spined sticklebacks in proportion to the concentration of effluent to which fish are exposed [17, 18,19], suggesting that a factor, or factors, associated with WWTW effluent may interfere with the function of the stress axis. Two endpoints have been used to quantify variation in stress axis responsiveness in this context in three-spined sticklebacks: stress-induced whole-body immunoreactive cortisol (WBIC) concentrations and the rate of cortisol release to water (CRTW). Both methods provide surrogates for the direct measurement of cortisol in the blood, a procedure which is impractical for small fish sampled at remote field sites. Measurement of WBIC is effective at discriminating between unstressed and stressed fish, but because WBIC quantifies cortisol in all compartments of the body the precise parity between cortisol levels in the blood and WBIC may not be consistent across time [26]. The release of cortisol to water, which occurs primarily from the blood across the gill epithelium [27], may be a more accurate surrogate of blood cortisol levels during stress than the multi-compartment total provided by WBIC. The rate of release of cortisol by this route has been shown to be proportional to the concentration of cortisol in the blood in a number of species of fish including three-spined sticklebacks [28]. The stress-induced WBIC concentration varies in relation to both natural and anthropogenic stressors [17,29] but the nature of the relationship between WBIC and effluent concentration differs from that of CRTW. Both WBIC and CRTW increase significantly in stressed fish, but the magnitude of the increase in WBIC is attenuated with increasing WWTW effluent concentrations (17) whereas the stress-induced increase in CRTW is amplified by exposure to increasing concentrations of effluent (18,19). On a mechanistic basis, the diametrically opposed responses to effluent exposure are not mutually exclusive and possible explanations for the different responses are discussed by Pottinger et al. [19].

The hypothesis that WWTW effluent affects function of the stress axis is consistent with reports that environmental contaminants can modify the activity of the stress axis in fish [9-15] and the fact that WWTW effluent represents an enriched source of anthropogenic chemicals [30,31]. However, the between-site range of responsiveness to a standardised stressor observed among sticklebacks captured at sites with no known upstream WWTW discharges is similar to that seen among fish from WWTW-contaminated sites. This suggests that the factor(s) responsible for modulation of the responsiveness of these fish to stressors is not uniquely associated with WWTW-contaminated sites. The results of the present investigation support this hypothesis by demonstrating that the reactivity of the stress axis of three-spined sticklebacks varies in proportion to indicators of water quality at sites unaffected by upstream WWTW inputs.

In the present study we exploited the availability of long-term water quality monitoring data for rivers in England and Wales (the Environment Agency WIMS chemical database) to provide a broad assessment of water quality at fish sampling sites with no identifiable point-source WWTW inputs. The possibility that some of the chemicals for which data were available have direct (or indirect) effects on somatic and endocrine endpoints among fish cannot be dismissed but we adopted this approach primarily on the basis that the water quality determinands provide a surrogate index for the presence of many other anthropogenic chemicals that occur together with those for which we have data, as components of the mixtures of contaminants that enter rivers and streams via diffuse and single-point routes [32,33].

Differences in physicochemical indicators of water quality between WWTW-contaminated and uncontaminated sites

The suite of water quality indicators for this exercise was selected pragmatically to provide coverage of factors indicative of anthropogenic inputs to the aquatic environment and comprised: water temperature [34]; pH, suspended solids [35], NO3 [36], NO2, NH3, orthophosphate (soluble reactive phosphorous) [37,38], Cl [39,40], Cu and Zn [30], Mg [41], and Si [42,43]. For all the determinands, with the exception of suspended solids, unionised NH_3, Cl and Cu, a significant difference in overall mean concentrations was evident between the WWTW-contaminated and uncontaminated sites. Differences between WWTW-contaminated and uncontaminated sites were most pronounced for NO_3, NO_2, orthophosphate, Si and Zn which in all cases were significantly higher at sites downstream of WWTW discharges, consistent with what is known of the dominant defining features of WWTW effluent [41]. Despite this overall trend, there was considerable overlap in the range of site means for concentrations of key water quality determinands, both within and between the contaminated and uncontaminated groups, indicating that those sites designated as uncontaminated by the absence of an upstream WWTW still received a range of contaminants via diffuse inputs [32,44], non-WWTW point sources, and/or undisclosed discharges such as unregistered septic tank systems [36,45,46].

Trends in fish size related to total dissolved NO_2 and NO_3, and WWTW effluent concentration, among sticklebacks at WWTW-contaminated and non-contaminated sites

There was considerable variation in fish size (mass and length) across sites and this variation was clearly linked to water quality at uncontaminated sites where mean fish size increased with increasing total oxidised N. Inorganic N is an important nutrient in freshwaters [47] and consequently higher total NO_2 and NO_3 concentrations tend to be associated with higher productivity in affected ecosystems, which is reflected in enhanced growth of resident fish [48]. At two sites (site 1, Old Eea Brook; and site 7, Town Brook) the fish were two to three times larger than those at other uncontaminated sites with similar total oxidised N concentrations. Inspection of the water quality data showed that concentrations of NO_2, unionised NH_3 and orthophosphate were atypically high for Town Brook (see Supplemental data, Table S1), but this was not the case for Old Eea brook where we must assume that other unidentified factors contributed to the size differential. At sites downstream of WWTW discharges the mean size of fish declined with increasing effluent concentration. This negative trend in fish size in relation to effluent concentration is consistent with the negative relationship between WWTW effluent concentration and size and growth rate of sticklebacks seen in a previous study [49] but is, superficially at least, not consistent with other studies in which a positive relationship was reported between effluent concentration and fish size [17,50,51]. However, in the 2013 study by Pottinger et al. [17] the effluent concentration range was lower than that in the current investigation and in the present study, fish captured at sites where the concentration of WWTW effluent was similar to that of the earlier study (30% or less) were significantly larger than the fish captured at uncontaminated sites (see Figure 2; GLM and Tukey's test, data not shown). This suggests that if WWTW effluent contains substances that directly impede growth processes in fish, negative effects may only be apparent when the concentration of effluent reaches a critical proportion of total river flow. At concentrations lower than this the net effect on growth is driven by nutrient enrichment. Certainly, many major components of WWTW effluent are potentially toxic to fish when present at high enough concentrations (e.g. NO_3; [47]) but in a complex mixture with individual components at relatively low concentrations, single agent effects are unlikely. It is more probable that observable toxicological effects of WWTW effluents [52-56], as distinct from the modulating effects of endocrine-active compounds, are a consequence of the aggregated impact of exposure to the complex mixture of persistent inorganic and organic chemicals present in wastewater effluent (33,57,58,59]. Effects on growth, manifested as a decline in mean population body size with increasing effluent concentration, presumably reflect the costs to the fish of coping with the chemical challenge.

Trends in stress responsiveness related to total dissolved NO_2 and NO_3 and water quality among sticklebacks at uncontaminated sites

A positive trend in stress-induced CRTW in relation to total N concentration among fish from sites with no known upstream WWTW inputs is reported here for the first time. The stress-induced CRTW for fish from uncontaminated sites shows an almost five-fold range in mean values, equivalent to the range of mean CRTW values seen for the WWTW-contaminated sites. The trend in cortisol release rates was positively related to declining water quality at both the uncontaminated and contaminated sites, despite there being opposing trends in fish size across the two groups. These relationships suggest that the factors affecting cortisol release rates at WWTW-contaminated sites may be independent of the factors affecting fish size. De-coupling of fish size and CRTW release rates is further supported by inspection of the data for two of the uncontaminated sites (site 1, Old Eea Brook; and site 7, Town Brook) for which the mean mass and length of the sampled fish were atypical compared to the size of fish at the other uncontaminated sites (Figure 2). In contrast, the cortisol release rate data for fish at these sites were entirely consistent with the overall trends seen across the uncontaminated sites (Figure 3).

In addition to the trends in fish size and CRTW in relation to total dissolved oxidised N among fish from uncontaminated sites, variation in a broad range of water chemistry determinands was shown to account for between 30% and 50% of variation in both body size and stress responsiveness, comparable to the 45% - 60% of variation in body size and stress responsiveness accounted for by water chemistry among fish from WWTW-contaminated sites. These outcomes, although explaining a substantial proportion of the variation in CRTW and fish size at both sets of sites, are probably quite conservative. The suite of water quality determinands comprised only eleven chemicals, together with pH, suspended solid loading and water temperature, and site selection and fish sampling was conducted before the water quality data were available so the distance between fish sampling site locations and water chemistry sampling sites varies (see Tables 1 and 2).

CONCLUSIONS

Variation in stress axis reactivity in fish exposed to WWTW effluent [17] has previously been attributed to one or more components of the complex mixture of micropollutants present in wastewater [33,57,58,59], based on the identity of chemicals known to interfere with the function of the stress axis in fish [10-15] and the likelihood that these are introduced into rivers via WWTW effluent. However, the present study suggests that a similar inter-population range of stress axis reactivity occurs at sites where there are no significant (known) upstream inputs of wastewater, implying that the causal factor(s) is/are not uniquely associated with WWTW effluent. These data suggest either that (1) the contaminants concerned are among the major water quality determinands known to be present in both WWTW-contaminated and uncontaminated rivers (NO_3, for example, may have endocrine-disrupting activity in fish [60-63]), or (2) that micropollutants, assumed to be primarily associated with treated wastewater inputs [57,59] are present within rivers uncontaminated by WWTW effluents to a greater degree than assumed. In either case, it will be necessary to investigate the mechanism underlying variation in stress axis reactivity between populations of sticklebacks, both to provide information on the nature of the causal factor(s) responsible for these effects and to better understand the functional implications of variations in the magnitude of the stress response at both the individual and population level.

SUPPLEMENTAL DATA

Table S1-S4; Figure S1

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Data availability – data are available from the authors on request

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Table 1. Sample sites with no identifiable upstream WWTW discharge. The sites are ranked by the mean total oxidised N concentration.

Sample site	Receiving water[a]	Sample NGR[b]	Total N (mg/l)[c]	EA site id[d]	EA site NGR[e]	Distance (m)[f]
1	Old Eea Brook	SJ 766 938	2.3 ± 0.1	88002075	SJ 766 938	0
2	Woodplumpton Brk (us)	SD 500 341	3.4 ± 0.2	88003855	SD 498 342	310
3	Barton Brook	SD 520 364	3.6 ± 0.2	88003850	SD 519 358	790
4	Woodplumpton Brk (ds)	SD 483 356	3.8 ± 0.2	88003867	SD 475 368	1380
5	New Draught	SD 479 401	4.2 ± 0.2	88003870	SD 479 401	0
6	Old River Brock	SD 480 401	4.7 ± 0.3	88003869	SD 480 401	0
7	Town Brook	SJ 695 991	5.7 ± 0.2	88002491	SJ 695 987	440
8	Black Brook	SD 366 137	7.5 ± 0.3	88003197	SD 375 157	2180
9	Lydiate Brook	SD 364 059	7.9 ± 0.3	88002882	SD 404 031	5240

[a]The river into which the WTW discharges and from which fish samples were collected

[b]The UK National Grid Reference for the fish sample location

[c]The average concentration of total oxidised N at each site derived from data in the EA WIMS database

[d]The identifier for the EA water quality sample location

[e]The National Grid Reference for the EA water quality sample site

[f]The approximate distance (m) between the EA water chemistry sample site and the fish sample site.

Table 2. Sample sites downstream of known WWTW discharges ranked by the estimated concentration of effluent at each site. The effluent concentration at each site was estimated as a percentage of the total flow using the LF2000-WQX model (Williams et al. 2009).

Sample site	WWTW[a]	Receiving water[b]	Population equivalent[c]	Treatment type[d]	% effluent concn.[e]	Sample location (NGR)[f]	EA site id[g]	EA site location (NGR)[h]	Distance (m)[i]
10	Leyland	R. Lostock	41526	TA2	19.4	SD 517 200	88003183	SD 508 197	1060
11	Royton	R. Irk	27590	TA1	24.9	SD 886 062	88002365	SD 872 059	1570
12	Tyldesley	Moss Brook	21771	TB2	25.3	SJ 689 985	88002500	SJ 680 983	1030
13	Blackburn	R. Darwen (ds)	120562	SB	30.1	SD 590 282	88003559	SD 595 288	1340
14	Glazebury	Glaze Brook	25462	SB	30.4	SJ 701 922	88002513	SJ 701 913	1010
15	Altrincham	Sinderland Brook	35426	TB2	31	SJ 738 905	88002519	SJ 712 904	2870
16	Darwen	R. Darwen (us)	30053	SB	34.3	SD 690 246	88003536	SD 688 239	810
17	Huyton	Netherley Brook	63234	SB	39	SJ 452 877	88002743	SJ 446 883	700
18	Denton	R. Tame	35050	SB	54	SJ 919 937	88002001	SJ 897 908	7160
19	Hillhouse	Maghull Hey Cop Drain	59952	TA2	95	SD 353 047	88009968	SD 337 039	1820

[a]The name of the upstream WWTW

[b]The river into which the WWTW discharges

[c]The unit of measure used to describe the size of a waste water discharge

[d]Ofwat (The UK Water Services Regulation Authority) sewage treatment classification codes: SB – Secondary biological; TA1- tertiary A1; TA2 – tertiary A2; TB2 – tertiary B2;

[e]The estimated concentration of effluent present as a percentage of total flow

[f]The UK National Grid Reference for the fish sample location; [g]the WIMS identifier for the EA water quality sample location; [h]the NGR for the EA water quality sample site

[i]The approximate distance (m) between the EA water chemistry sample site and the fish sample site.

Table 3. Mean values (± SEM) for water quality determinands at sites affected by upstream WWTW discharges (WWTW-contaminated) and sites with no identifiable upstream WWTW inputs (uncontaminated). Significant variation across sites was detected for all the variables (GLM; p < 0.001) but only the outcomes for the comparison of WWTW-contaminated and uncontaminated sites for each determinand are shown. All data © Environment Agency 2015

	WWTW-contaminated		**uncontaminated**		**ANOVA**	
Determinand	**n**	**Mean ± SEM**	**n**	**Mean ± SEM**	**(df) *F***	***p***
Water temp. (^{o}C)	3075	11.06 ± 0.08	1985	10.54 ± 0.11	(1,5041) 23.8	< 0.001
Suspended solids (mg/l)	3048	22.67 ± 0.67	1915	20.68 ± 1.01	(1,4944) 2.2	0.14
Dissolved O_2 (mg/l)	3017	9.17 ± 0.05	1889	9.38 ± 0.06	(1,4887) 39.4	< 0.001
pH	3078	7.52 ± 0.01	1990	7.68 ± 0.01	(1,5049) 228	< 0.001
Total NH_3-N (mg/l)	3076	1.2011 ± 0.03	1978	1.2003 ± 0.06	(1,5035) 15.2	< 0.001
Unionised NH_3-N (mg/l)	2288	0.007 ± 0.0002	1804	0.008 ± 0.0006	(1,4073) 0.2	0.65
NO_3-N (mg/l)	3045	6.20 ± 0.07	1918	4.54 ± 0.08	(1,4944) 590	< 0.001
NO_2-N (mg/l)	6071	0.20 ± 0.002	1969	0.12 ± 0.004	(1,8021) 182	< 0.001
Orthophosphate (mg/l)	3041	1.37 ± 0.02	1913	0.66 ± 0.02	(1,4935) 857	< 0.001
Cl (mg/l)	2953	55.66 ± 0.77	1804	52.59 ± 2.06	(1,4738) 0.41	0.52
Si (mg/l)	1080	8.96 ± 0.08	315	5.25 ± 0.15	(1,1383) 164	< 0.001
Mg (mg/l)	1559	10.03 ± 0.13	490	13.1 ± 0.21	(1,2030) 4.6	0.033
Cu (µg/l)	1230	5.62 ± 0.35	518	5.31 ± 0.1	(1,1729) 2.7	0.10
Zn (µg/l)	1688	35.97 ± 0.55	503	18.5 ± 0.76	(1,2179) 34.7	< 0.001

Table 4. The outcomes of multiple linear regressions conducted between fourteen water quality parameters (water temperature, suspended solids, dissolved O_2, pH, NH_3, un-ionised NH_3, NO_3, NO_2, orthophosphate, Cl, reactive Si, Mg, Cu, Zn) and somatic (mass, length, condition, K) and endocrine (CRTW; stress-induced cortisol release to water) data for three-spined sticklebacks at ten sites downstream of WWTW discharges (WWTW) and at nine sites with no identifiable upstream WWTW discharge (non-WWTW).

		Regression		**ANOVA**	
Group	**y-variable**	r^2	r^2**-adj**	**(df) F**	p
WWTW	Mass (mg)	0.52	0.50	(8,183) 24.8	< 0.001
	Length (mm)	0.61	0.59	(8,183) 35.9	< 0.001
	CRTW (pg/g/h)	0.50	0.45	(8,91) 11.2	< 0.001
Non-WWTW	Mass (mg)	0.52	0.49	(7,164) 24.9	< 0.001
	Length (mm)	0.53	0.51	(7,164) 26.2	< 0.001
	CRTW (pg/g/h)	0.33	0.27	(7,78) 5.6	< 0.001

Figure legends

Figure 1. Total oxidised N (a) and total orthophosphate (b) at sites uncontaminated by WWTW effluent (open boxes, sites 1-9) and WWTW-contaminated sites (grey boxes, sites 10-19). Uncontaminated sites are plotted in rank order by total oxidised N concentration and WWTW-contaminated sites are ranked by the estimated effluent concentration (% of total river flow). The 25th and 75th percentiles are indicated by the lower and upper boundaries of each box, the error bars indicate the 10th and 90th percentiles, and the 5th and 95th percentiles are denoted by the symbols. The line within the box denotes the median. Boxes sharing the same letter are not significantly different from each other (p > 0.05).

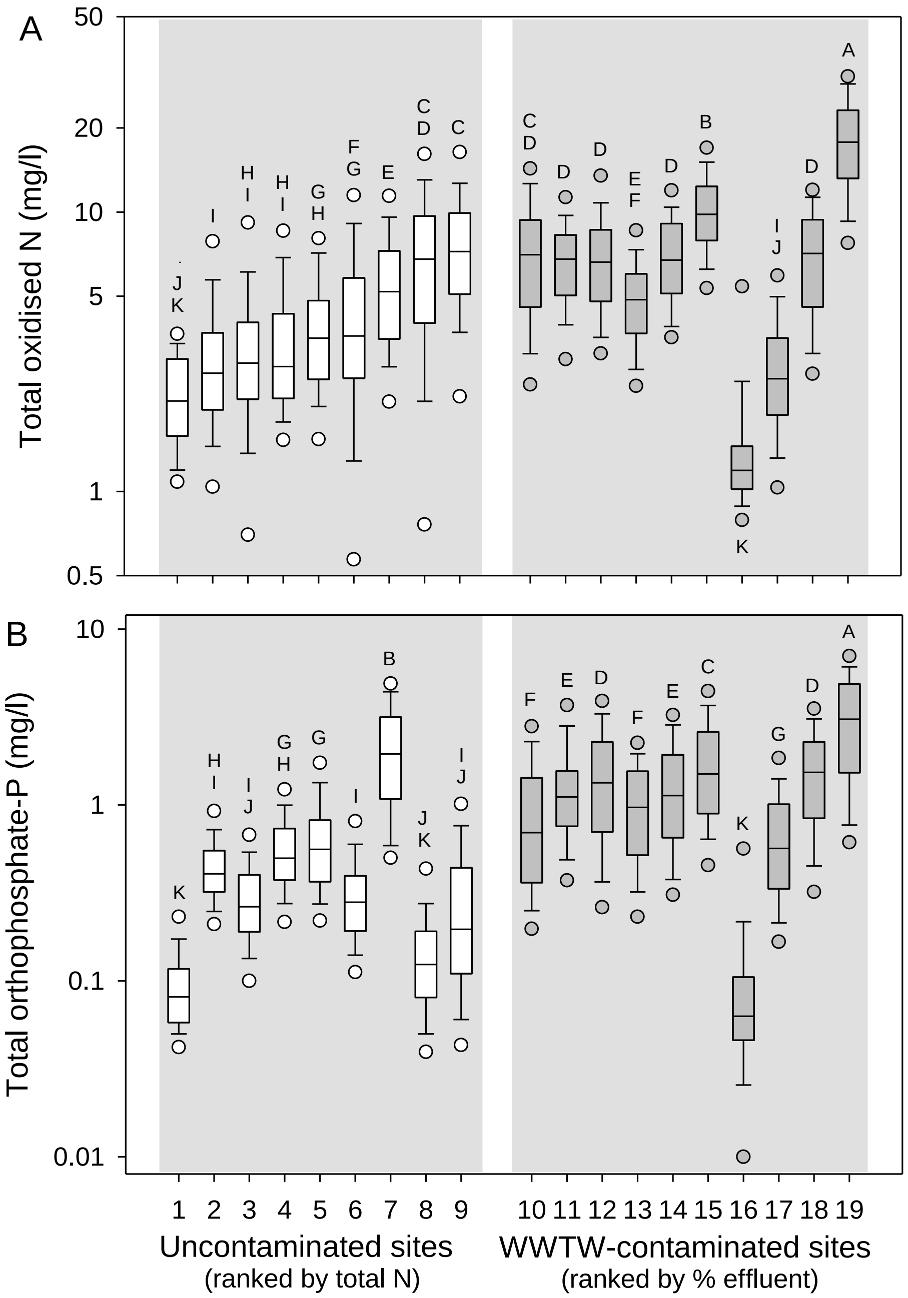


Figure 2. The mass (a) and length (b) of three-spined sticklebacks captured at each site and ranked by total oxidised N (WWTW-uncontaminated sites) and by estimated effluent concentration (% of total river flow; WWTW-contaminated sites). Each point is the mean ± SEM (n = 11 - 21). Spearman rank order correlations were significant ($p < 0.001$) in all cases. The best-fit linear regression lines are shown to highlight significant trends. The regressions were conducted using the raw data but means and standard errors for each site are shown for clarity. Site 1 (Old Eea Brook) and site 7 (Town Brook) were excluded from the regressions.

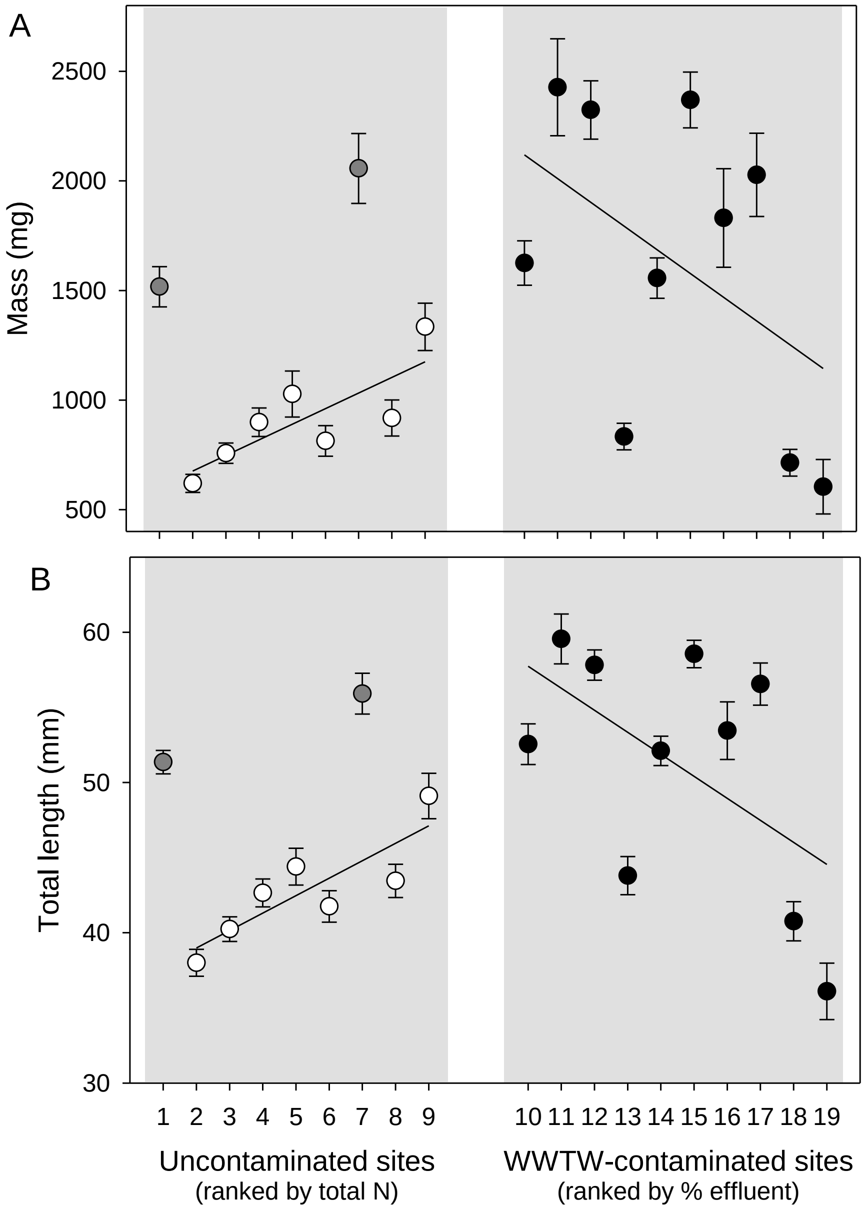
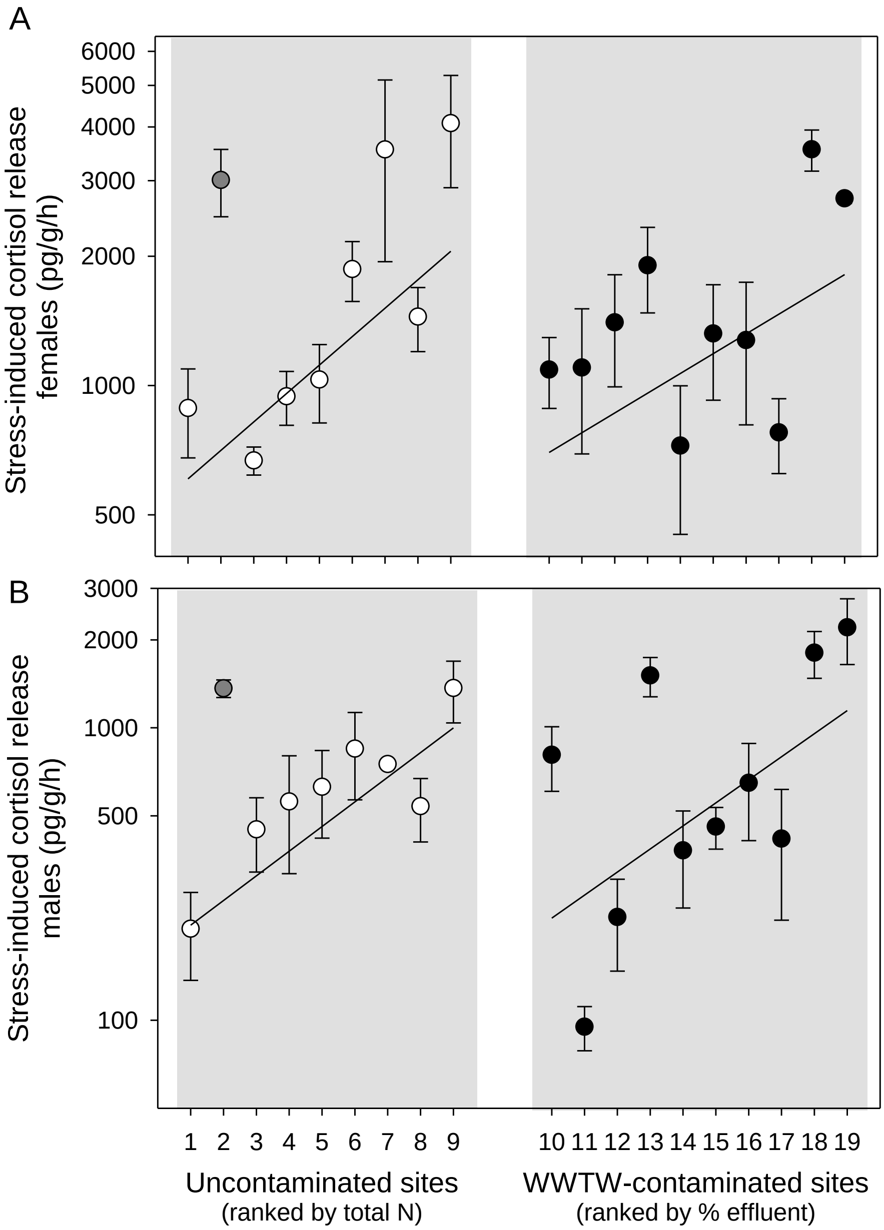


Figure 3. The stress-induced rate of release of cortisol to water by (a) female and (b) male sticklebacks captured at each site plotted against sample sites ranked by total oxidised N (WWTW-uncontaminated sites) and by estimated effluent concentration (% of total river flow; WWTW-contaminated sites). Each point is the mean ± SEM (n = 3 - 8). Spearman rank order correlations were significant ($p < 0.001$ uncontaminated sites; $p < 0.01$ contaminated sites) in all cases. The best-fit linear regression lines are shown to highlight significant trends. The regressions were conducted using the raw data but means and standard errors for each site are shown for clarity. Site 2 was omitted from the regression



SUPPLEMENTARY DATA

Table S1. Summary of chemistry data retrieved from the EA WIMS database. All data © Environment Agency 2016

Receiving water	Sample site	EA site id	Determinand	n	Mean	SE	Max.	Min.	Median	Start date	End date	Duration (yrs)
Old Eea Brook	1	88002075	pH	177	7.383	0.0168	8.1	6.4	7.4	12/02/1992	08/12/2006	14.8
			Water temperature (oC)	176	11.107	0.329	23	2	11	12/02/1992	08/12/2006	14.8
			Dissolved oxygen (mg/l)	180	6.066	0.159	14	0.69	6	12/02/1992	08/12/2006	14.8
			Ammoniacal Nitrogen as N (mg/l)	177	1.797	0.203	13.8	0.03	0.892	12/02/1992	08/12/2006	14.8
			Nitrogen, Total Oxidised as N (mg/l)	147	2.311	0.101	12.6	0.5	2.11	13/07/1994	08/12/2006	12.4
			Nitrate as N (mg/l)	177	2.079	0.0939	12.432	0.05	1.95	12/02/1992	08/12/2006	14.8
			Nitrite as N (mg/l)	176	0.127	0.00966	1.25	0.02	0.0895	12/02/1992	08/12/2006	14.8
			Ammonia un-ionised as N (mg/l)	174	0.0144	0.00366	0.505	0.0001	0.004	12/02/1992	08/12/2006	14.8
			Solids, Suspended (mg/l)	177	11.305	1.257	129	2	7	12/02/1992	08/12/2006	14.8
			Chloride (mg/l)	177	79.467	6.168	1020	8	66	12/02/1992	08/12/2006	14.8
			Orthophosphate, reactive as P (mg/l)	177	0.0996	0.00491	0.396	0.02	0.0812	12/02/1992	08/12/2006	14.8
			Magnesium (mg/l)	23	12.648	1.068	24.2	1.4	11.5	17/02/1994	14/12/1995	1.8
			Copper (ug/l)	24	3.476	0.416	7.6	1	2.67	17/01/1994	14/12/1995	1.9
			Zinc (ug/l)	24	39.238	5.924	116	5	28.65	17/01/1994	14/12/1995	1.9
Woodplumpton Brk (us)	2	88003855	pH	207	7.719	0.0198	8.92	6.7	7.73	23/09/1980	21/11/2006	26.2
			Water temperature (oC)	206	10.24	0.326	23	1	10.15	23/09/1980	21/11/2006	26.2
			Dissolved oxygen (mg/l)	208	9.51	0.164	15.7	2.3	9.9	23/09/1980	21/11/2006	26.2
			Ammoniacal Nitrogen as N (mg/l)	207	0.53	0.0772	12.5	0.03	0.249	23/09/1980	21/11/2006	26.2
			Nitrogen, Total Oxidised as N (mg/l)	147	3.44	0.245	25.3	0.2	2.65	06/06/1994	21/11/2006	12.5
			Nitrate as N (mg/l)	207	3.32	0.182	25.2	0.191	2.7	23/09/1980	21/11/2006	26.2
			Nitrite as N (mg/l)	207	0.08	0.00725	1.3	0.00853	0.0625	23/09/1980	21/11/2006	26.2
			Ammonia un-ionised as N (mg/l)	184	0.00	0.00051	0.0561	0.0001	0.00215	26/02/1990	21/11/2006	16.7

			Solids, Suspended (mg/l)	206	19.97	2.175	243	2	9	23/09/1980	21/11/2006	26.2
			Chloride (mg/l)	207	56.67	12.64	2580	14.1	35.7	23/09/1980	21/11/2006	26.2
			Orthophosphate, reactive as P (mg/l)	207	0.46	0.0147	1.5	0.01	0.406	23/09/1980	21/11/2006	26.2
			Si (mg/l)	33	6.11	0.392	9.2	1.64	6.75	27/04/1988	26/04/1993	5.0
			Magnesium (mg/l)	21	11.45	0.481	16	8.21	11.4	13/04/1994	11/12/1995	1.7
			Copper (ug/l)	21	6.26	0.505	11.1	3.07	5.75	17/02/1994	11/12/1995	1.8
			Zinc (ug/l)	23	27.60	4.418	99.2	6.8	22.1	19/01/1994	11/12/1995	1.9
Barton Brook	3	88003850	pH	217	7.965	0.0264	9.9	7.1	7.9	23/09/1980	05/02/2007	26.4
			Water temperature (oC)	217	10.406	0.361	26.2	0	10	23/09/1980	05/02/2007	26.4
			Dissolved oxygen (mg/l)	139	11.562	0.176	22.7	5.9	11.3	08/06/1994	05/02/2007	12.7
			Ammoniacal Nitrogen as N (mg/l)	217	0.5	0.0529	4.84	0.01	0.247	23/09/1980	05/02/2007	26.4
			Nitrogen, Total Oxidised as N (mg/l)	149	3.552	0.218	22	0.2	2.88	06/07/1994	05/02/2007	12.6
			Nitrate as N (mg/l)	217	3.417	0.163	21.9	0.196	2.802	23/09/1980	05/02/2007	26.4
			Nitrite as N (mg/l)	217	0.0933	0.00657	0.68	0.004	0.067	23/09/1980	05/02/2007	26.4
			Ammonia un-ionised as N (mg/l)	194	0.0058	0.000795	0.0965	0.0002	0.0029	26/02/1990	05/02/2007	17.0
			Solids, Suspended (mg/l)	216	20.023	3.525	420	2	8	23/09/1980	05/02/2007	26.4
			Chloride (mg/l)	216	30.861	0.934	135	8	28.05	23/09/1980	05/02/2007	26.4
			Orthophosphate, reactive as P (mg/l)	217	0.311	0.0118	1.23	0.037	0.264	23/09/1980	05/02/2007	26.4
			Si (mg/l)	39	4.274	0.414	7.75	0.1	5	27/04/1988	26/04/1993	5.0
			Magnesium (mg/l)	36	9.737	0.45	13.7	3.34	10.03	13/04/1994	05/09/2003	9.4
			Copper (ug/l)	42	4.87	0.396	11.4	2.19	3.895	08/06/1994	05/09/2003	9.2
			Zinc (ug/l)	7	5.679	0.679	9.75	5	5	18/07/2001	13/12/2001	0.4
Woodplumpton Brk (ds)	4	88003867	pH	205	7.728	0.0241	9.44	6.7	7.7	23/09/1980	21/11/2006	26.2
			Water temperature (oC)	205	10.34	0.336	25	1	10	23/09/1980	21/11/2006	26.2
			Dissolved oxygen (mg/l)	205	9.59	0.158	18.1	3.4	9.65	23/09/1980	21/11/2006	26.2

			Ammoniacal Nitrogen as N (mg/l)	205	0.79	0.057	5.5	0.08	0.51	23/09/1980	21/11/2006	26.2
			Nitrogen, Total Oxidised as N (mg/l)	146	3.78	0.241	25.5	1.09	2.8	06/06/1994	21/11/2006	12.5
			Nitrate as N (mg/l)	205	3.55	0.184	25.4	0.98	2.69	23/09/1980	21/11/2006	26.2
			Nitrite as N (mg/l)	205	0.14	0.00703	0.562	0.025	0.1	23/09/1980	21/11/2006	26.2
			Ammonia un-ionised as N (mg/l)	182	0.01	0.000553	0.0403	0.00031	0.00535	26/02/1990	21/11/2006	16.7
			Solids, Suspended (mg/l)	204	24.96	4.747	814	2	10.5	23/09/1980	21/11/2006	26.2
			Chloride (mg/l)	205	53.25	7.429	1400	16	37.5	23/09/1980	21/11/2006	26.2
			Orthophosphate, reactive as P (mg/l)	205	0.59	0.0229	2.45	0.146	0.498	23/09/1980	21/11/2006	26.2
			Si (mg/l)	32	5.30	0.43	8.8	0.52	6.21	27/04/1988	26/04/1993	5.0
			Magnesium (mg/l)	21	10.84	0.518	15.1	5.69	11.5	13/04/1994	11/12/1995	1.7
			Copper (ug/l)	22	5.20	0.38	9.19	3.15	4.68	17/02/1994	11/12/1995	1.8
			Zinc (ug/l)	23	18.58	3.216	70.6	5.82	11.9	20/01/1994	11/12/1995	1.9
New Draught	5	88003870	pH	295	7.709	0.0198	9.27	6.8	7.7	23/09/1980	22/11/2012	32.2
			Water temperature (oC)	296	10.396	0.287	25	0	10	23/09/1980	22/11/2012	32.2
			Dissolved oxygen (mg/l)	295	9.589	0.112	14.9	4.6	9.6	23/09/1980	22/11/2012	32.2
			Ammoniacal Nitrogen as N (mg/l)	287	0.506	0.0377	5.6	0.02	0.304	23/09/1980	22/11/2012	32.2
			Nitrogen, Total Oxidised as N (mg/l)	220	4.198	0.198	26.9	0.87	3.54	06/07/1994	22/11/2012	18.4
			Nitrate as N (mg/l)	286	4.128	0.159	26.8	0.841	3.464	23/09/1980	22/11/2012	32.2
			Nitrite as N (mg/l)	287	0.115	0.00517	0.528	0.018	0.091	23/09/1980	22/11/2012	32.2
			Ammonia un-ionised as N (mg/l)	265	0.00371	0.000252	0.0272	0.00025	0.00239	26/02/1990	22/11/2012	22.8
			Solids, Suspended (mg/l)	287	20.056	1.807	230	2	11	23/09/1980	22/11/2012	32.2
			Chloride (mg/l)	227	38.148	1.633	231	15.1	33.2	23/09/1980	21/11/2007	27.2
			Orthophosphate, reactive as P (mg/l)	252	0.681	0.0294	2.91	0.05	0.559	23/09/1980	22/11/2012	32.2
			Si (mg/l)	179	5.427	0.197	9.76	0.19	6.23	27/04/1988	21/11/2007	19.6
			Magnesium (mg/l)	184	11.2	0.199	18.6	4.71	11.5	16/02/1994	22/11/2012	18.8
			Copper (ug/l)	191	5.64	0.193	28.1	0.768	4.9	16/02/1994	22/11/2012	18.8

			Zinc (ug/l)	226	14.744	0.978	179	5	11.1	19/01/1994	22/11/2012	18.9
Old R. Brock	6	88003869	pH	204	7.67	0.0182	8.35	6.8	7.7	21/08/1985	18/10/2007	22.2
			Water temperature (oC)	203	10.269	0.322	22	0.5	10	21/08/1985	18/10/2007	22.2
			Dissolved oxygen (mg/l)	204	9.52	0.173	18.6	3.3	9.64	21/08/1985	21/11/2006	21.3
			Ammoniacal Nitrogen as N (mg/l)	203	0.446	0.0528	5.25	0.01	0.205	21/08/1985	21/11/2006	21.3
			Nitrogen, Total Oxidised as N (mg/l)	146	4.716	0.33	27.3	0.2	3.605	06/07/1994	21/11/2006	12.4
			Nitrate as N (mg/l)	203	4.376	0.259	27.2	0.1	3.51	21/08/1985	21/11/2006	21.3
			Nitrite as N (mg/l)	203	0.0939	0.00594	0.79	0.004	0.07	21/08/1985	21/11/2006	21.3
			Ammonia un-ionised as N (mg/l)	182	0.00333	0.00054	0.0556	0.0001	0.00132	26/02/1990	21/11/2006	16.7
			Solids, Suspended (mg/l)	202	19.139	3.777	670	1	7	21/08/1985	21/11/2006	21.3
			Chloride (mg/l)	203	48.481	4.339	851	11	38	21/08/1985	21/11/2006	21.3
			Orthophosphate, reactive as P (mg/l)	203	0.345	0.0185	1.93	0.05	0.28	21/08/1985	21/11/2006	21.3
			Si (mg/l)	32	4.508	0.402	7.9	0.1	5.35	27/04/1988	26/04/1993	5.0
			Magnesium (mg/l)	20	9.922	0.535	13.7	3.32	9.98	16/02/1994	18/10/2007	13.7
			Copper (ug/l)	22	4.009	0.349	7.21	1.72	3.69	16/02/1994	03/04/2006	12.1
			Zinc (ug/l)	23	20.154	2.77	53.2	5	15.5	19/01/1994	03/04/2006	12.2
Town Brook	7	88002491	pH	282	7.522	0.0147	8.6	6.66	7.525	14/08/1985	10/03/2010	24.6
			Water temperature (oC)	282	11.159	0.249	22	2.5	11.1	14/08/1985	10/03/2010	24.6
			Dissolved oxygen (mg/l)	282	8.251	0.111	15.5	0.896	8.3	14/08/1985	10/03/2010	24.6
			Ammoniacal Nitrogen as N (mg/l)	281	4.875	0.327	31.2	0.03	2.78	14/08/1985	10/03/2010	24.6
			Nitrogen, Total Oxidised as N (mg/l)	178	5.741	0.227	24.2	1.1	5.195	17/07/1994	10/03/2010	15.7
			Nitrate as N (mg/l)	281	4.347	0.171	18.2	0.2	3.32	14/08/1985	10/03/2010	24.6
			Nitrite as N (mg/l)	280	0.247	0.0253	6.01	0.0245	0.14	14/08/1985	10/03/2010	24.6
			Ammonia un-ionised as N (mg/l)	228	0.0311	0.00359	0.542	0.00021	0.00647	23/01/1990	10/03/2010	20.1
			Solids, Suspended (mg/l)	245	24.827	2.573	450	2	15	14/08/1985	19/03/2007	21.6

			Chloride (mg/l)	246	49.315	2.835	626	12	44.85	14/08/1985	19/03/2007	21.6
			Orthophosphate, reactive as P (mg/l)	280	2.266	0.0896	10.9	0.05	1.95	14/08/1985	10/03/2010	24.6
			Magnesium (mg/l)	44	16.085	0.848	41.4	3.46	15.75	24/02/1994	12/01/2004	9.9
			Copper (ug/l)	48	4.32	0.185	8.5	2.54	4.09	27/01/1994	12/01/2004	10.0
			Zinc (ug/l)	36	22.259	2.083	64.7	8.18	17	27/01/1994	03/12/2001	7.9
Black Brook	8	88003197	pH	220	7.689	0.0296	9.15	6.88	7.6	01/07/1993	22/11/2012	19.4
			Water temperature (oC)	220	11.242	0.353	23.1	0.6	11	01/07/1993	22/11/2012	19.4
			Dissolved oxygen (mg/l)	200	10.617	0.149	17.2	5	10.6	25/04/1995	22/11/2012	17.6
			Ammoniacal Nitrogen as N (mg/l)	219	0.199	0.0169	1.95	0.03	0.139	01/07/1993	22/11/2012	19.4
			Nitrogen, Total Oxidised as N (mg/l)	214	7.486	0.333	31.1	0.2	6.79	14/04/1994	22/11/2012	18.6
			Nitrate as N (mg/l)	218	7.439	0.329	30.938	0.168	6.73	01/07/1993	22/11/2012	19.4
			Nitrite as N (mg/l)	216	0.074	0.00339	0.34	0.004	0.0595	01/07/1993	22/11/2012	19.4
			Ammonia un-ionised as N (mg/l)	217	0.00142	0.000109	0.0154	0.00025	0.001	01/07/1993	22/11/2012	19.4
			Solids, Suspended (mg/l)	220	18.606	1.404	195	2	14	01/07/1993	22/11/2012	19.4
			Chloride (mg/l)	165	57.341	1.316	134	19	54	01/07/1993	18/07/2011	18.1
			Orthophosphate, reactive as P (mg/l)	190	0.168	0.0152	2.4	0.02	0.124	01/07/1993	18/07/2011	18.1
			Magnesium (mg/l)	119	17.602	0.349	27	9.3	16.9	21/12/1994	22/11/2012	17.9
			Copper (ug/l)	122	5.684	0.177	14.2	1.73	5.565	26/01/1994	22/11/2012	18.8
			Zinc (ug/l)	122	15.904	0.987	78.2	5	13.4	26/01/1994	22/11/2012	18.8
Lydiate Brook	9	88002882	pH	183	7.761	0.0278	9.59	5.5	7.8	22/01/1992	17/03/2010	18.2
			Water temperature (oC)	180	9.437	0.282	17.6	1.1	9	22/01/1992	17/03/2010	18.2
			Dissolved oxygen (mg/l)	164	10.625	0.123	19	6.8	10.4	22/01/1992	17/03/2010	18.2
			Ammoniacal Nitrogen as N (mg/l)	182	0.142	0.0261	4.17	0.03	0.0795	22/01/1992	17/03/2010	18.2
			Nitrogen, Total Oxidised as N (mg/l)	153	7.886	0.332	23.4	0.75	7.22	30/06/1994	17/03/2010	15.7
			Nitrate as N (mg/l)	181	8.133	0.307	23.3	0.735	7.42	22/01/1992	17/03/2010	18.2

			Nitrite as N (mg/l)	178	0.0597	0.00341	0.34	0.004	0.0465	22/01/1992	17/03/2010	18.2
			Ammonia un-ionised as N (mg/l)	178	0.00168	0.000427	0.0539	0.00001	0.0007	22/01/1992	17/03/2010	18.2
			Solids, Suspended (mg/l)	158	27.008	4.908	590	3	12.5	22/01/1992	27/11/2007	15.9
			Chloride (mg/l)	158	72.098	8.181	1120	19	51.55	22/01/1992	27/11/2007	15.9
			Orthophosphate, reactive as P (mg/l)	182	0.336	0.0294	2.88	0.033	0.197	22/01/1992	17/03/2010	18.2
			Magnesium (mg/l)	22	11.325	0.733	21.6	7.31	10.5	11/04/1994	15/12/1995	1.7
			Copper (ug/l)	26	5.695	0.393	11.8	2.93	5.555	14/02/1994	01/07/2002	8.4
			Zinc (ug/l)	26	29.302	5.226	106	6.86	20.75	17/01/1994	01/07/2002	8.5
R. Lostock	10	88003183	pH	256	7.877	0.0209	9.03	6	7.87	24/09/1985	30/11/2012	27.2
			Water temperature (oC)	253	10.915	0.277	27	0.7	11	24/09/1985	30/11/2012	27.2
			Dissolved oxygen (mg/l)	235	10.361	0.131	18.7	0.1	10.3	24/09/1985	30/11/2012	27.2
			Ammoniacal Nitrogen as N (mg/l)	259	0.649	0.0625	6.89	0.03	0.294	24/09/1985	30/11/2012	27.2
			Nitrogen, Total Oxidised as N (mg/l)	215	7.341	0.252	26	1.25	7.03	27/04/1994	30/11/2012	18.6
			Nitrate as N (mg/l)	257	7.082	0.22	25.9	0.06	6.7	24/09/1985	30/11/2012	27.2
			Nitrite as N (mg/l)	254	0.127	0.00853	1.05	0.00898	0.08	24/09/1985	30/11/2012	27.2
			Ammonia un-ionised as N (mg/l)	234	0.00732	0.000889	0.136	0.00016	0.00275	20/01/1992	30/11/2012	20.9
			Solids, Suspended (mg/l)	258	20.605	2.911	560	2	9	24/09/1985	30/11/2012	27.2
			Chloride (mg/l)	232	39.496	1.189	182	17.4	36	24/09/1985	22/10/2012	27.1
			Orthophosphate, reactive as P (mg/l)	241	1.031	0.0583	5.76	0.116	0.696	24/09/1985	22/10/2012	27.1
			Si (mg/l)	29	7.311	0.457	10.2	1.06	7.9	30/07/2008	22/10/2012	4.2
			Magnesium (mg/l)	109	12.262	0.253	18.6	6.07	12.3	10/05/1993	17/11/2004	11.5
			Copper (ug/l)	109	4.917	0.382	39.5	1.14	4	10/05/1993	30/11/2012	19.6
			Zinc (ug/l)	127	28.793	3.613	397	6.03	17.6	10/05/1993	30/11/2012	19.6
R. Irk	11	88002365	pH	254	7.683	0.018	8.65	6.5	7.72	07/08/1985	01/12/2006	21.3
			Water temperature (oC)	245	10.69	0.228	19	2.9	11	07/08/1985	01/12/2006	21.3

			Dissolved oxygen (mg/l)	251	9.83	0.139	14.2	1.6	10.3	07/08/1985	01/12/2006	21.3
			Ammoniacal Nitrogen as N (mg/l)	252	1.456	0.144	15.1	0.03	0.546	07/08/1985	01/12/2006	21.3
			Nitrogen, Total Oxidised as N (mg/l)	149	6.77	0.187	12.5	2.13	6.79	10/07/1994	01/12/2006	12.4
			Nitrate as N (mg/l)	250	6.38	0.149	12.9	1.52	6.345	07/08/1985	01/12/2006	21.3
			Nitrite as N (mg/l)	247	0.2	0.0088	0.719	0.02	0.16	07/08/1985	01/12/2006	21.3
			Ammonia un-ionised as N (mg/l)	198	0.00735	0.00088	0.138	0.0002	0.00405	25/01/1990	01/12/2006	16.9
			Solids, Suspended (mg/l)	254	21.484	2.826	548	2	9	07/08/1985	01/12/2006	21.3
			Chloride (mg/l)	252	47.917	2.682	598	15.5	39	07/08/1985	01/12/2006	21.3
			Orthophosphate, reactive as P (mg/l)	251	1.349	0.0596	5.4	0.062	1.11	07/08/1985	01/12/2006	21.3
			Magnesium (mg/l)	45	9.86	0.372	13.6	3.85	10.2	03/02/1993	05/07/2002	9.4
			Copper (ug/l)	51	11.466	7.972	410	2.15	3.26	03/02/1993	05/07/2002	9.4
			Zinc (ug/l)	51	29.389	3.361	136	9.26	24.5	03/02/1993	05/07/2002	9.4
Moss Brook	12	88002500	pH	302	7.255	0.0142	8.6	6.47	7.255	14/08/1985	21/11/2012	27.3
			Water temperature (oC)	301	11.34	0.232	21	3.1	11.4	14/08/1985	21/11/2012	27.3
			Dissolved oxygen (mg/l)	300	7.603	0.133	13.5	1.1	8.06	14/08/1985	21/11/2012	27.3
			Ammoniacal Nitrogen as N (mg/l)	300	2.419	0.139	11.11	0.03	1.595	14/08/1985	21/11/2012	27.3
			Nitrogen, Total Oxidised as N (mg/l)	179	6.9	0.223	17.2	0.2	6.53	17/07/1994	21/11/2012	18.4
			Nitrate as N (mg/l)	281	6.428	0.178	24	0.182	5.95	14/08/1985	21/11/2012	27.3
			Nitrite as N (mg/l)	298	0.205	0.0108	1.05	0.018	0.14	14/08/1985	21/11/2012	27.3
			Ammonia un-ionised as N (mg/l)	248	0.00835	0.000707	0.0695	0.0001	0.00303	23/01/1990	21/11/2012	22.8
			Solids, Suspended (mg/l)	300	34.137	2.438	465	2	23.25	14/08/1985	21/11/2012	27.3
			Chloride (mg/l)	247	48.983	2.081	471	13	47.6	14/08/1985	21/11/2012	27.3
			Orthophosphate, reactive as P (mg/l)	282	1.624	0.0701	5.75	0.098	1.335	14/08/1985	21/11/2012	27.3
			Magnesium (mg/l)	139	16.838	0.426	35.8	4.49	16.7	24/02/1994	21/11/2012	18.8
			Copper (ug/l)	141	4.461	0.189	19.5	2.04	3.93	27/01/1994	21/11/2012	18.8
			Zinc (ug/l)	139.0	33.266	1.216	93.2	11.7	29.6	27/01/1994	21/11/2012	18.8

R. Darwen (ds)	13	88003559	pH	197	7.825	0.0159	8.9	7.05	7.81	19/09/1985	18/06/2008	22.8
			Water temperature (oC)	202	11.554	0.294	23.2	0.5	11.2	19/09/1985	18/06/2008	22.8
			Dissolved oxygen (mg/l)	196	10.494	0.101	14.3	6	10.4	19/09/1985	18/06/2008	22.8
			Ammoniacal Nitrogen as N (mg/l)	199	0.814	0.0461	4.4	0.05	0.65	19/09/1985	18/06/2008	22.8
			Nitrogen, Total Oxidised as N (mg/l)	151	4.973	0.155	12.6	1.1	4.82	21/04/1994	18/06/2008	14.2
			Nitrate as N (mg/l)	200	4.679	0.13	12.3	0.5	4.56	19/09/1985	18/06/2008	22.8
			Nitrite as N (mg/l)	198	0.131	0.00631	0.57	0.03	0.111	19/09/1985	18/06/2008	22.8
			Ammonia un-ionised as N (mg/l)	67	0.0111	0.00146	0.0702	0.0008	0.0069	20/02/1991	04/03/1999	8.0
			Solids, Suspended (mg/l)	199	21.157	1.544	163	5	15	19/09/1985	18/06/2008	22.8
			Chloride (mg/l)	199	57.069	2.606	335	11.9	53	19/09/1985	18/06/2008	22.8
			Orthophosphate, reactive as P (mg/l)	198	1.082	0.0464	2.96	0.14	0.968	19/09/1985	18/06/2008	22.8
			Si (mg/l)	25	6.766	0.331	9.77	3.29	7.36	10/07/1986	09/02/1993	6.6
			Magnesium (mg/l)	126	8.075	0.242	26.4	3.07	8.3	20/02/1991	02/06/2005	14.3
			Copper (ug/l)	38	11.737	1.726	68.4	2.52	8.555	21/04/1994	18/06/2008	14.2
			Zinc (ug/l)	82	26.055	1.688	93.2	7.5	23.15	20/02/1991	18/06/2008	17.3
Glaze Brook	14	88002513	pH	290	7.361	0.0165	9.7	6.5	7.37	14/08/1985	10/03/2010	24.6
			Water temperature (oC)	313	11.258	0.266	22.7	2.2	11	14/08/1985	26/10/2012	27.2
			Dissolved oxygen (mg/l)	303	6.697	0.155	12.6	1.5	6.9	14/08/1985	26/10/2012	27.2
			Ammoniacal Nitrogen as N (mg/l)	306	1.75	0.0999	8.7	0.03	1.065	14/08/1985	26/10/2012	27.2
			Nitrogen, Total Oxidised as N (mg/l)	208	7.162	0.176	16	2.69	6.735	11/04/1994	26/10/2012	18.6
			Nitrate as N (mg/l)	305	6.203	0.136	14.33	0.52	5.75	14/08/1985	26/10/2012	27.2
			Nitrite as N (mg/l)	287	0.275	0.0155	2.2	0.0296	0.2	14/08/1985	26/10/2012	27.2
			Ammonia un-ionised as N (mg/l)	135	0.00982	0.00133	0.149	0.0001	0.0051	23/01/1990	10/03/2010	20.1
			Solids, Suspended (mg/l)	294	22.607	1.431	205	2	16	14/08/1985	26/10/2012	27.2
			Chloride (mg/l)	294	70.289	2.499	310	20	59.4	14/08/1985	26/10/2012	27.2

			Orthophosphate, reactive as P (mg/l)	302	1.394	0.0546	5.03	0.129	1.13	14/08/1985	26/10/2012	27.2
			Si (mg/l)	37	8.622	0.428	13.4	1.3	9.08	26/09/1985	26/10/2012	27.1
			Magnesium (mg/l)	107	19.389	0.398	28.2	8.8	19.6	13/03/1986	06/02/2004	17.9
			Copper (ug/l)	97	4.946	0.136	11.6	2.71	4.79	13/04/2000	26/10/2012	12.5
			Zinc (ug/l)	147	31.984	1.383	115	7.8	28.6	26/09/1985	26/10/2012	27.1
Sinderland Brk	15	88002519	pH	323	7.372	0.0176	8.6	3.9	7.4	09/10/1985	03/12/2012	27.2
			Water temperature (oC)	319	11.703	0.245	22.3	2	11.7	09/10/1985	03/12/2012	27.2
			Dissolved oxygen (mg/l)	318	8.149	0.113	17.9	2.4	8.13	09/10/1985	03/12/2012	27.2
			Ammoniacal Nitrogen as N (mg/l)	319	1.315	0.0757	9.3	0.03	0.92	09/10/1985	03/12/2012	27.2
			Nitrogen, Total Oxidised as N (mg/l)	220	10.324	0.239	23.1	1.9	9.815	15/07/1994	03/12/2012	18.4
			Nitrate as N (mg/l)	316	9.867	0.194	23	0.5	9.369	09/10/1985	03/12/2012	27.2
			Nitrite as N (mg/l)	315	0.311	0.0155	2.32	0.0257	0.22	09/10/1985	03/12/2012	27.2
			Ammonia un-ionised as N (mg/l)	270	0.0061	0.000467	0.0682	0.00001	0.00385	02/01/1990	03/12/2012	22.9
			Solids, Suspended (mg/l)	322	24.23	1.995	430	2	15.25	09/10/1985	03/12/2012	27.2
			Chloride (mg/l)	319	51.445	1.368	348	22	48.1	09/10/1985	03/12/2012	27.2
			Orthophosphate, reactive as P (mg/l)	318	1.882	0.0721	6.95	0.184	1.5	09/10/1985	03/12/2012	27.2
			Si (mg/l)	54	9.456	0.204	12.3	6.17	9.13	23/07/2008	03/12/2012	4.4
			Magnesium (mg/l)	140	9.972	0.15	14	4.56	10.1	11/02/1994	03/12/2012	18.8
			Copper (ug/l)	144	5.387	0.205	24.5	1.63	4.82	14/01/1994	03/12/2012	18.9
			Zinc (ug/l)	143	27.041	1.034	84.9	11.2	24.5	14/01/1994	03/12/2012	18.9
R. Darwen (us)	16	88003536	pH	291	7.56	0.0155	8.54	6.07	7.57	19/09/1985	04/12/2012	27.2
			Water temperature (oC)	290	9.939	0.209	18	0.0001	10	19/09/1985	04/12/2012	27.2
			Dissolved oxygen (mg/l)	288	10.642	0.0892	17.5	3.6	10.6	19/09/1985	04/12/2012	27.2
			Ammoniacal Nitrogen as N (mg/l)	288	0.234	0.0246	3	0.005	0.101	19/09/1985	04/12/2012	27.2
			Nitrogen, Total Oxidised as N (mg/l)	182	1.593	0.108	11.6	0.43	1.19	01/07/1994	04/12/2012	18.4

			Nitrate as N (mg/l)	287	1.339	0.07	11.4	0.05	1.1	19/09/1985	04/12/2012	27.2
			Nitrite as N (mg/l)	286	0.0506	0.00461	0.88	0.006	0.0282	19/09/1985	04/12/2012	27.2
			Ammonia un-ionised as N (mg/l)	227	0.00148	0.000152	0.0207	0.00005	0.0008	02/01/1990	04/12/2012	22.9
			Solids, Suspended (mg/l)	259	16.29	1.72	376	2	9	19/09/1985	18/06/2008	22.8
			Chloride (mg/l)	258	35.724	2.15	400	9	29.3	19/09/1985	18/06/2008	22.8
			Orthophosphate, reactive as P (mg/l)	305	0.146	0.0188	3.39	0.01	0.063	19/09/1985	04/12/2012	27.2
			Si (mg/l)	61	8.052	0.172	10.9	5	8.14	10/07/1986	04/05/1993	6.8
			Magnesium (mg/l)	24	13.117	1.092	20.5	3.98	12.3	01/07/1991	30/12/1997	6.5
			Copper (ug/l)	30	5.364	1.039	26.1	1.52	3.425	10/06/1994	04/12/2012	18.5
			Zinc (ug/l)	25	32.972	4.167	82.1	5.3	32.3	11/03/1992	27/04/1998	6.1
Netherley Brook	17	88002743	pH	162	7.676	0.0175	8.6	6.9	7.7	19/02/1992	03/08/2005	13.5
			Water temperature (oC)	162	11.188	0.25	21.2	4	11	19/02/1992	03/08/2005	13.5
			Dissolved oxygen (mg/l)	151	6.895	0.154	11.4	2.8	6.9	19/02/1992	03/08/2005	13.5
			Ammoniacal Nitrogen as N (mg/l)	162	1.385	0.104	11.7	0.05	1.025	19/02/1992	03/08/2005	13.5
			Nitrogen, Total Oxidised as N (mg/l)	136	2.91	0.137	11.5	0.503	2.525	05/07/1994	03/08/2005	11.1
			Nitrate as N (mg/l)	162	2.675	0.129	11.4	0.05	2.335	19/02/1992	03/08/2005	13.5
			Nitrite as N (mg/l)	161	0.165	0.0072	0.679	0.032	0.141	19/02/1992	03/08/2005	13.5
			Ammonia un-ionised as N (mg/l)	160	0.0166	0.00166	0.183	0.0002	0.0117	19/02/1992	03/08/2005	13.5
			Solids, Suspended (mg/l)	162	21.951	4.1	550	2	8.5	19/02/1992	03/08/2005	13.5
			Chloride (mg/l)	162	69.619	4.764	510	6.1	53	19/02/1992	03/08/2005	13.5
			Orthophosphate, reactive as P (mg/l)	161	0.721	0.0426	3.1	0.06	0.566	19/02/1992	03/08/2005	13.5
			Magnesium (mg/l)	33	15.558	0.88	21.9	2.44	16.3	25/02/1994	20/06/2000	6.3
			Copper (ug/l)	36	4.978	0.346	12.8	2.34	4.515	11/01/1994	20/06/2000	6.4
			Zinc (ug/l)	26	31.348	5.652	119	8.61	20	11/01/1994	23/05/2000	6.4
R. Tame	18	88002001	pH	946	7.456	0.00729	8.9	6.1	7.47	27/01/1984	20/12/2012	28.9

			Water temperature (oC)	933	10.938	0.153	23	1	10.5	27/01/1984	20/12/2012	28.9
			Dissolved oxygen (mg/l)	919	9.983	0.0875	71.3	1.67	10	27/01/1984	20/12/2012	28.9
			Ammoniacal Nitrogen as N (mg/l)	934	0.958	0.0332	7.55	0.03	0.56	27/01/1984	20/12/2012	28.9
			Nitrogen, Total Oxidised as N (mg/l)	477	7.103	0.135	14.4	1.57	7.11	01/07/1994	23/11/2012	18.4
			Nitrate as N (mg/l)	930	6.3	0.0953	17.9	0.5	5.931	27/01/1984	23/11/2012	28.8
			Nitrite as N (mg/l)	933	0.22	0.0052	1.1	0.0178	0.18	27/01/1984	20/12/2012	28.9
			Ammonia un-ionised as N (mg/l)	701	0.00415	0.0002	0.0939	0	0.003	05/01/1990	20/12/2012	23.0
			Solids, Suspended (mg/l)	943	21.43	1.174	401	1.3	11	27/01/1984	20/12/2012	28.9
			Chloride (mg/l)	933	62.281	1.543	890	5	56.5	27/01/1984	23/11/2012	28.8
			Orthophosphate, reactive as P (mg/l)	926	1.66	0.0347	12.4	0.05	1.53	27/01/1984	23/11/2012	28.8
			Si (mg/l)	869	9.12	0.0858	23.75	0.2	9.2	27/01/1984	23/11/2012	28.8
			Magnesium (mg/l)	781	6.845	0.0459	14.4	2.1	7.04	19/08/1985	15/01/2004	18.4
			Copper (ug/l)	527	5.569	0.164	78.5	1	5.16	19/01/1990	20/12/2012	22.9
			Zinc (ug/l)	891	41.439	0.713	185	5	37.5	27/01/1984	20/12/2012	28.9
Maghull Hey Cop	19	88009968	pH	57	7.348	0.0481	8	6.5	7.4	05/01/2000	27/08/2008	8.6
Drain			Water temperature (oC)	57	12.526	0.504	19.1	5.9	12.7	05/01/2000	27/08/2008	8.6
			Dissolved oxygen (mg/l)	56	9.463	0.461	33	6	9.05	05/01/2000	05/06/2008	8.4
			Ammoniacal Nitrogen as N (mg/l)	57	2.282	0.724	38.5	0.03	1.01	05/01/2000	27/08/2008	8.6
			Nitrogen, Total Oxidised as N (mg/l)	57	18.388	0.954	34.6	1.36	17.8	05/01/2000	27/08/2008	8.6
			Nitrate as N (mg/l)	57	18.268	0.952	34.5	1.29	17.7	05/01/2000	27/08/2008	8.6
			Nitrite as N (mg/l)	57	0.127	0.0377	1.97	0.013	0.05	05/01/2000	27/08/2008	8.6
			Ammonia un-ionised as N (mg/l)	48	0.00599	0.00128	0.0472	0.0005	0.00303	06/12/2000	27/08/2008	7.7
			Solids, Suspended (mg/l)	57	21.956	2.338	89	3	19	05/01/2000	27/08/2008	8.6
			Chloride (mg/l)	57	69.902	1.81	99.3	41.1	68.7	05/01/2000	27/08/2008	8.6
			Orthophosphate, reactive as P (mg/l)	57	3.259	0.264	7.44	0.137	3.07	05/01/2000	27/08/2008	8.6
			Magnesium (mg/l)	55	15.637	0.395	28.3	9.45	15.5	05/01/2000	27/08/2008	8.6

	Copper (ug/l)	57	3.189	0.161	7.61	1.53	3.05	05/01/2000	27/08/2008	8.6
	Zinc (ug/l)	57	29.396	1.463	66.6	10.4	30.3	05/01/2000	27/08/2008	8.6

Table S2. Summary of somatic data for male sticklebacks captured at each site. K = coefficient of condition = (100 x mass)/(length^3).

				Mass (mg)		Length (mm)		K	
sample site	**Receiving water**	**WWTW**	**n**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**
1	Old Eea Brook	-	10	1482	88	51.1	1.0	1.101	0.020
2	Woodplumpton Brk (us)	-	7	487	60	34.9	1.3	1.125	0.055
3	Barton Brook	-	10	647	52	38.8	1.1	1.087	0.022
4	Woodplumpton Brk (ds)	-	6	976	155	43.2	2.3	1.151	0.036
5	New Draught	-	7	701	81	39.7	1.4	1.093	0.040
6	Old R. Brock	-	11	840	103	42.1	1.7	1.085	0.028
7	Town Brook	-	7	1885	166	54.0	1.6	1.180	0.040
8	Black Brook	-	7	1147	123	47.3	1.5	1.062	0.040
9	Lydiate Brook	-	10	1296	124	48.7	1.9	1.086	0.031
10	R. Lostock	LEYLAND	7	1380	207	49.1	3.1	1.099	0.037
11	R. Irk	ROYTON	9	2372	212	59.8	1.8	1.088	0.041
12	Moss Brook	TYLDESLEY	7	1859	72	54.3	0.7	1.162	0.036
13	R. Darwen (ds)	BLACKBURN	10	843	87	43.8	1.8	0.981	0.035
14	Glaze Brook	GLAZEBURY	11	1544	117	51.6	1.0	1.102	0.034
15	Sinderland Brook	ALTRINCHAM	9	2027	110	56.8	1.0	1.099	0.019
16	R. Darwen (us)	DARWEN	7	1497	162	50.6	1.9	1.128	0.030
17	Netherley Brook	HUYTON	11	1488	76	52.3	0.9	1.034	0.021
18	R. Tame	DENTON	6	779	55	42.8	1.1	0.986	0.030
19	Maghull Hey Cop Drain	HILLHOUSE	11	747	218	38.2	3.2	1.081	0.043

Table S3. Summary of somatic data for female sticklebacks captured at each site. K = coefficient of condition = (100 x mass)/(length^3).

				Mass (mg)		Length (mm)		K	
sample site	**Receiving water**	**WWTW**	**n**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**
1	Old Eea Brook	-	10	1552	165	51.6	1.3	1.092	0.039
2	Woodplumpton Brk (us)	-	13	691	45	39.7	0.9	1.096	0.033
3	Barton Brook	-	11	858	62	41.6	1.1	1.178	0.023
4	Woodplumpton Brk (ds)	-	14	866	68	42.4	1.0	1.102	0.021
5	New Draught	-	13	1203	133	46.9	1.3	1.116	0.044
6	Old R. Brock	-	9	782	95	41.3	1.3	1.065	0.032
7	Town Brook	-	4	2359	299	59.3	1.6	1.114	0.063
8	Black Brook	-	13	795	95	41.4	1.2	1.073	0.044
9	Lydiate Brook	-	10	1372	184	49.5	2.5	1.065	0.042
10	R. Lostock	LEYLAND	13	1757	96	54.4	1.0	1.087	0.027
11	R. Irk	ROYTON	11	2473	373	59.4	2.7	1.085	0.041
12	Moss Brook	TYLDESLEY	9	2686	136	60.6	1.0	1.204	0.037
13	R. Darwen (ds)	BLACKBURN	10	824	90	43.8	1.9	0.955	0.031
14	Glaze Brook	GLAZEBURY	8	1573	157	52.8	2.0	1.057	0.038
15	Sinderland Brook	ALTRINCHAM	11	2650	175	60.0	1.4	1.217	0.031
16	R. Darwen (us)	DARWEN	13	2011	330	55.0	2.7	1.103	0.028
17	Netherley Brook	HUYTON	9	2688	288	61.8	1.7	1.108	0.039
18	R. Tame	DENTON	11	678	89	39.6	1.9	1.041	0.055
19	Maghull Hey Cop Drain	HILLHOUSE	9	430	41	33.6	1.0	1.104	0.027

Table S4. Linear regression equations (Sigmaplot v. 13; Systat Software Inc.) for lines fitted to the data in Figs 2 and 3 in order to visualise trends.

Fig.	**Site classification**	**Parameter**	r^2	***F***	***p***
2	Uncontaminated	Mass	0.18	$F(1,139) = 30.4$	< 0.001
		Length	0.23	$F(1,139) = 41.0$	< 0.001
	WWTW-contaminated	Mass	0.12	$F(1,190) = 25.0$	< 0.001
		Length	0.18	$F(1,190) = 43.0$	< 0.001
3	Uncontaminated	CRTW (female)	0.22	$F(1,43) = 12.3$	< 0.01
		CRTW (male	0.23	$F(1,29) = 8.7$	< 0.01
	WWTW-contaminated	CRTW (female)	0.18	$F(1,50) = 11.1$	< 0.01
		CRTW (male)	0.21	$F(1,46) = 12.4$	< 0.001

Figure S1. The coefficient of condition [K, (100 x mass)/(length3)] for (a) male and (b) female three-spined sticklebacks captured at each site and ranked by total oxidised N (WWTW-uncontaminated sites) and by estimated effluent concentration (% of total river flow; WWTW-contaminated sites). Each point is the mean ± SEM (n = 11 - 21). The best-fit linear regression lines are shown but Spearman rank order correlations were not significant (p > 0.14) for any case. The regressions were conducted using the raw data but means and standard errors for each site are shown for clarity.